STUDIES ON THE EFFECT OF CAPSAICIN ON HUMAN ERYTHROCYTES

THESIS

SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

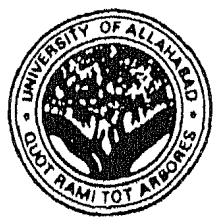
IN THE
DEPARTMENT OF BIOCHEMISTRY

FACULTY OF SCIENCE

UNIVERSITY OF ALLAHABAD

BY
SUAIB LUQMAN
M.Sc.

UNDER THE SUPERVISION OF
DR. SYED IBRAHIM RIZVI



2002
DEPARTMENT OF BIOCHEMISTRY
UNIVERSITY OF ALLAHABAD
ALLAHABAD – 211002
INDIA

Dedicated to

* *My Father*

  For uncompromising principles that guided my life to reach higher goals.

* *My Mother*

  For leading her children into intellectual persuits, her magnificent devotion and co-operation with constant inspiration for making everything worthwhile.

* *My Siblings*

  For their abundant support, patience, understanding and for their love.

* *My Teachers*

  For showing me the excitement and joy of research.

ACKNOWLEDGEMENT

With profound sense of gratitude I am highly thankful to my supervisor Dr. Syed Ibrahim Rizvi, for all that I have been able to achieve. I pay my obeisance and adulation to him for the pains he took to advice me and making my humble effort a success. His constructive criticism, corrections and help in carrying out this investigation have boosted my morale to work hard in the right direction. His inspiring guidance not only created the scientific temper in me but also instilled in me the confidence that has enabled me to complete this work.

I record my profound thanks to Dr. K.K. Sharma, Head, Department of Biochemistry, University of Allahabad for the help and co-operation during the course of this study.

I am immensely grateful to the authorities of the University for providing necessary facilities rendered during the course of this study.

I express my gratitude to all the staff members for their encouragement and help throughout the work.

I acknowledge with a deep sense of indebtness and appreciation, the help provided by Dr. A.K. Rastogi Dy. Director & Head, Biochemistry Division, Central Drug Research Institute, Lucknow and Prof. Krishna Misra, Co-ordinator, Centre for Biotechnology, University of Allahabad.

My sincere thanks are forwarded to the library authorities of National Medical Library, New Delhi, Central Drug Research Institute, Lucknow, and National Botanical Research Institute, Lucknow for their kind co-operation.

My warmest thanks are due to my colleagues Dr. M.A. Zaid, Mr. Navneet Kumar, Ms. Rafat Anis & Mrs. Neetu Mishra for their encouragement, helpful criticism and unfailing support throughout the work.

At last I pay my special thanks to my friends for their constant help and encouragement in all circumstances. I also extend my thanks to many others who have helped me during my study and whom I may not have mentioned.

My thanks are also due to University Grants Commission and Council of Scientific and Industrial Research, New Delhi for financial assistance.

(SUAIB LUQMAN)

UNIVERSITY OF ALLAHABAD

FACULTY OF SCIENCE

DEPARTMENT OF BIOCHEMISTRY

CERTIFICATE OF ORIGINAL WORK

This is to certify that the present thesis attached herewith entitled "**Studies on the Effect of Capsacin on Human Erythrocytes**", prepared and submittted by Mr. Suaib Luqman, in fulfilment of the requirement for the Degree of Doctor of Philosophy, is a bonafide record of the research work done by him under my supervision and guidance.

(Dr. Syed Ibrahim Rizvi)
Supervisor
Senior Lecturer
Department of Biochemistry

TABLE OF CONTENT *Page No.*

1.4.3 Neurotoxicity by vanilloids

1.5 Blood: Composition and physiological functions

1.5.1 Human Erythrocyte

1.5.2 The Red Cell Membrane

1.5.2.1 Structure of the red cell membrane

1.5.2.2 Composition of the red cell membrane.

1.5.2.3 Red cell membrane proteins

1.5.2.3 Red cell membrane lipids

1.5.2.4 Red cell membrane carbohydrate

1.5.2.5 Red cell membrane fluidity.

1.5.3 The red cell metabolism

1.5.3.1 Intracellular metabolites and their importance

1.5.3.2 ATP : The prime source of energy.

1.5.3.3 Oxidized and reduced glutathione

1.5.4 Enzymes responsible for maintaining redox state of the red cell

1.5.4.1 Glutathione reductase

1.5.4.2 Glutathione peroxidase

1.5.4.3 Catalase

3.1.4 pH measurement

3.1.5 Optical measurements

3.1.6 Blood Collection

3.1.7 Isolation of erythrocytes

3.1.8 Isolation of red blood cell ghost

3.2 Methods followed

3.2.1 Determination of the ghost membrane protein content

3.2.2 Preparation of red cell hemolysate

3.2.3 Estimation of hemoglobin

3.2.4 Determination of acetylcholinesterase activity

3.2.5 Assay of membrane sodium-potasium adenosine triphosphatase (Na^+/K^+-ATPase) activity.

3.2.6 Assay of membrane calcium adenosine triphosphatase (Ca^{++}-ATPase) activity.

3.2.7 Estimation of erythrocyte intracellular reduced glutathione (GSH) content.

3.2.8 Determination of erythrocyte membrane sulfhydryl (SH) groups.

3.2.9 Determination of red cell osmotic fragility.

3.2.10 Experiments with capsaicin and / or capsazepine.

LIST OF TABLES

Page No.

LIST OF FIGURES

Page No.

1. INTRODUCTION

1.1 THE WORLD OF SPICES

India is considered to be the "home of spices". Spices are aromatic vegetable products of tropical origin that are used primarily for seasoning or garnishing foods and beverages. They are characterized by odour, pungency and bitter taste. However, it is not only the taste and culinary compulsions that have made use of spices popular but also they are good appetizers and are considered essential in the culinary art all over the world. Some of the spices possess anti-oxidant properties (e.g. *Carcuma longa*), while others are used as preservatives in pickles and chutneys etc. Some spices also possess strong anti-microbial and anti-biotic activity (e.g. Cloves contain a chemical called eugenol that inhibits the growth of bacteria).

Most of the spices possess medicinal properties and have a profound effect on human health. For instance, spices intensify salivary flow and the secretion of amylase, neuraminic acid and hexosamines. They favour cleansing of the oral cavity, check infection and caries and protect the mucous membrane against thermic, mechanical and chemical irritation. Spices also activate the adreno-cortical function and fortify resistance and physical capacity. Stroke and blood pressure can be markedly diminished or augmented by means of spices. Spices inhibit thrombus formation and accelerate thrombolysis. All these important physiological and medicinal aspects of spices deserve our serious consideration and research (Pruthi, 1987).

There are about 70 species of spices grown in different parts of the world. Many of these are grown in India. Spices may comprise different plant components or parts as shown in **Table 1.** (Kochhar, 1995).

TABLE - 1

	Name of the spice	Part of the Plant Used
1.	Cloves, Saffron	Floral parts
2.	Cardamom, Chillies	Fruits
3.	Black pepper, Juniper	Berries
4.	Caraway, Coriander	Seeds
5.	Ginger, Turmeric	Rhizomes
6.	Angelica, Lovage	Roots
7.	Bay leaves, Majoram, Mints	Leaves
8.	Nutmegs	Kernel
9.	Onion, Garlic	Bulbs
10.	Mace	Aril
11.	Cinnamon, Cassia	Bark

1.2 THE STORY OF CAPSICUM

In general, Chilli is a common word and capsicum is the botanical term. Capsicum refers to the fruits of several species belonging to Solanaceae family.

The term CAPSICUM is derived either from the Greek word "Kapso" meaning to bite or from the Latin "Capsa" meaning box. Capsicum is especially popular in hot climates where its normal dietary intake may be as high as 2.5 grams per day (corresponding to approximately 1 mg capsaicin per kg body weight, daily) (Rumsfield and West, 1991).

Hot peppers have been used as a condiment as well as for food. They are used as flavoring agents, and coloring material in cookery. It is employed as an additive to native foods. Tabasco sauce is made by pickling the pulp in strong vinegar and / or brine. Oil of pepper is valued as a flavouring for sausages, canned meats, soups, table sauces, certain beverages and liquors. *Capsicum frutescens* is used in the manufacture of sauces and curry powders and also in preparations of pickles. *Capsicum annuum*, the green non-pungent form are eaten raw as salads. The colouring matter of the ripe fruit consists of several compounds such as - Capsanthin, capsorubin, zeaxanthin, lutein, cryptoxanthin, α and β carotenes and a few unidentified xanthophylls. Capsaicin, the pungent ingredient of capsicum pods is used in the manufacture of ginger ale and ginger beer. Medicinally capsicum peppers have been used internally in the past as a powerful stimulant and carminative but externally as a counter-irritant to cure rheumatism. The aclinary uses are too numerous to mention but some of the uses are as follows.

1.2.1 Traditional and current uses of capsicum

Capsicum is known for stimulating the circulation and altering temperature regulation and is also used in high fevers. Applied to the skin, capsicum paste, desensitizes nerve endings and it has been used in the past as a

local analgesic. The capsicidins found in the seeds of capsicum fruits are believed to have anti-biotic properties. The heating quality of capsicum makes it a valuable remedy for poor circulation and related conditions. In particular, it improves blood flow to the hands and feet and to the central organs. The uses of capsicum in traditional medicines are multifold (Nadkarni, 1976).

It is also rubefacient, increasing blood flow to the affected parts and this helps to stimulate the circulation in 'cold' rheumatic and arthritic conditions, aiding the removal of waste products and increasing the flow of nutrients to the tissues. A paste of capsicum is used locally in tonsilities. In diphtheria its application believed to hasten cure. Chillies are taken to relieve wind and colic and to stimulate secretion of the digestive juices, thereby aiding digestion. A pinch of chilly powder is excellent in gargles for sore throats. Finally, extraordinary as it may seem, chillies are useful for some types of diarrhoea. Capsicum in combination with cinchona is used in gout and advanced stages of rheumatism. Capsicum has also been used as a remedy for snake-bite (Whittet, 1968; Lembeck, 1987).

1.2.2 Composition of capsicum

The composition of Indian and American red and green chillies are given in **Table 2**. Green chillies are rich in Vitamin A and C and the seed contains traces of starch (Sayed and Bhagavandas, 1980). The fruit also contains oil, coloring matter which is non-pungent and yield 20 - 25 per cent alcoholic extract.

TABLE - 2

COMPOSITION OF CULTIVARS OF CHILLIES

Physico-Chemical Characteristics	Indian green chilli	Indian red chilli (dry)	Indian paprika (dried)	American pepper/ chilli	American red pepper
Moisture %	82.60	10.00	7.90	6.50	6.20
Protein %	2.90	15.90	13.80	14.00	16.00
Fat %	0.60	6.20	10.40	14.10	15.50
Fibre %	6.80	30.20	19.20	15.60	26.00
Carbohydrates %	6.10	31.60	41.10	42.60	28.30
Total Ash %	1.00	6.10	7.60	7.20	8.00
Calcium %	0.03	0.16	0.20	0.10	0.10
Phosphorus %	0.08	0.37	0.30	0.32	0.32
Iron %	1.20	2 30	0.23	0.01	0.01
Sodium %	0.06	—	0.02	0.01	0.01
Potassium %	0.21	—	2.40	2.10	2.10
Thiamine (mg%)	0.19	—	0.60	0.59	0.52
Riboflavin (mg%)	1.18	—	1.36	1.66	0.93
Niacin (mg%)	0.50	—	15.3	14.20	13.60
Ascorbic acid (mg%)	111.00	50.0	58.80	63.70	29.41
Vitamin A(1U/100g)	54	576	4915	6165	3530
Calorific value (Cal/ 100g)	41	246	390	415	420

(Pruthi, J.S. 1987)

1.3 CHEMISTRY OF THE VANILLOIDS

1.3.1 CAPSAICIN

1.3.1.1. The pungent principle

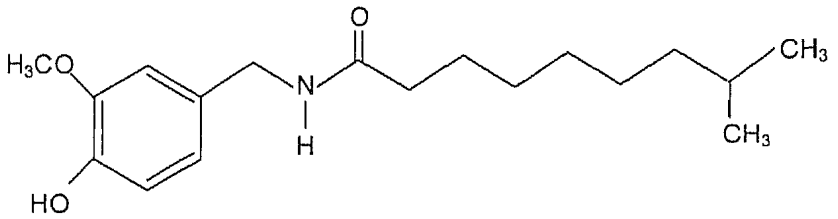
The pungent principle in chillies is an alkaloid (vanilloid) called capsaicin. Capsaicin (**Figure 1**) is a crystalline condensation product of 3-hydroxy-4-methoxy benzylamine and decylenic acid which produces a highly irritating vapour on heating. Capsaicin retains its pungency in a dilution of one in a million parts of water. Its content varies from 0.0 - 1.8 per cent in different capsicum species. Commercial chilli samples contain about 0.1 per cent capsaicin.

1.3.1.2 Properties

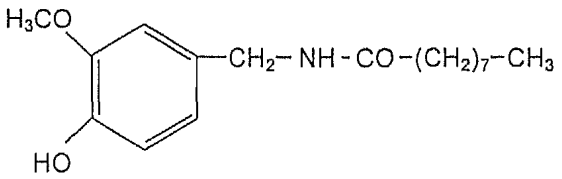
The chemical properties of capsaicin has been extensively investigated. (Govindrajan, 1986). It is weakly acidic, and contains a phenolic group as shown in **Figure 1.**

Formula	:	$C_{18}H_{27}O_3N$
Formula weight	:	305.4
Nomenclature	:	trans 8-methyl-N-vanillyl-6-nonenamide
Melting point	:	64.0° - 65°C
Boiling point	:	210° - 220°C
Solubility	:	Capsaicin is sparingly soluble in cold water and more soluble in boiling water but readily soluble in organic solvent like ethanol, acetone and chloroform.

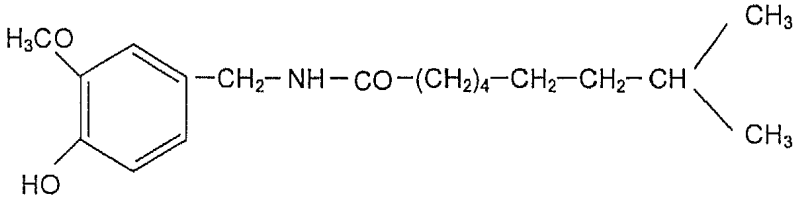
FIGURE 1



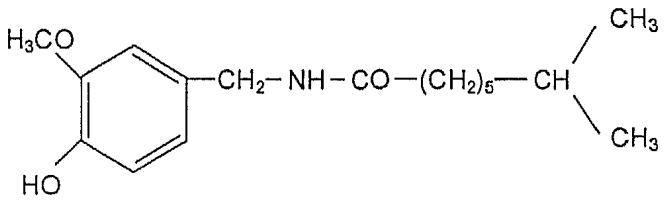
CAPSAICIN



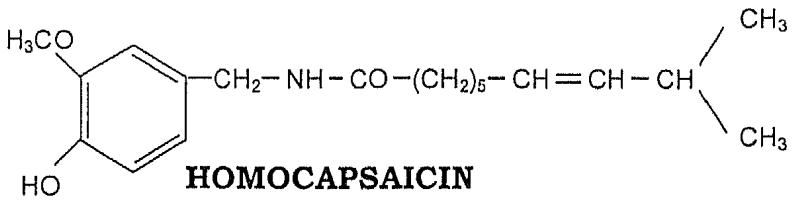
SYNTHETIC CAPSAICIN



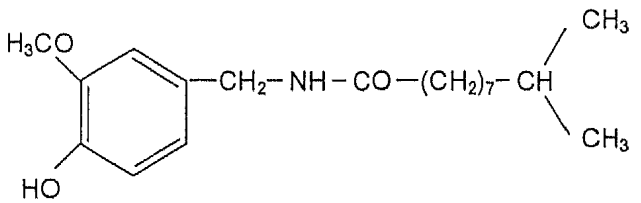
DIHYDROCAPSAICIN



NORDIHYDROCAPSAICIN



HOMOCAPSAICIN



HOMODIHYDROCAPSAICIN

It is not precipitated, or only very slightly, by the usual reagents which precipitate alkaloids. The phenolic group can be methylated to form methyl capsaicin, a derivative with much less pungency than the parent compound. Its pungency is not destroyed by heating with 2% NaOH, but is destroyed by oxidation with $KMnO_4$ or $K_2Cr_2O_7$. Synthetic capsaicin, (Vanillyl - nonanoylamide) which has a saturated side chain is not readily oxidized. The structures of natural capsaicin and the most commonly used synthetic analog are shown in **Figure 1**. In addition to capsaicin, four naturally occuring derivatives has also been isolated and identified. These are shown in **Figure 1.**

1.3.1.3 Units to measure pungency of capsaicin

The pungency of capsaicin is measured in SCOVILLE HEAT UNITS (SHU). In 1912, Wilbur Scoville calibrated the potency of pepper by extracting capsicum in alcohol and diluting it untill pungency was just detected after placing a drop on his tongue. The threshold concentrations of capsaicin that produce a lukewarm and painful, burning sensation on the tongue are 2×10^7 and $> 10^{-6}$ g/ml respectively (Szolcsanyi, 1982). Nowadays, the pungency of chillies with their capsaicin content are analysed by using HPLC technique. (Weaver, 1984). Other methods used to measure pungency include colorimetry, which however, gives errors due to interference by other coloring principles. Thin layer chromatography has also been used (Karawya, 1967).

SHU of some of the chillies with their content- capsaicin, dihydrocapsaicin and oleoresin are given in **Table No. 3.**

TABLE - 3

Capsaicin and Dihydrocapsaicin content and pungency in different varieties of Indian red chillies.

Capsicum Type	Oleoresin (% w/w)	Capsaicin (% w/w)	Dihydrocapsaicin/ (% w/w)	Pungency (SHU)
Tezpur	15.0	4.28	1.42	855000
Guntur	12.0	0.20	0.16	53250
Gwalior	12.5	0.43	0.23	112500
Kashmir	11.0	0.18	0.09	40500
Patna	19.1	0.44	0.21	96000

(Mathur *et al.* 2000)

1.3.1.4 Tezpur variety : The hottest chilli

Tezpur chilli variety of India is the hottest chilli known so far. Prior to this, Red Savina Habanero, a Mexican variety was reported to be the hottest chilli in the world having a pungency of 577000 SHU (Mathur *et al.* 2000).

1.3.2 Resiniferatoxin : An analog of capsaicin

Apart from capsaicin, there are a number of other natural and synthetic pungent compounds, including zingerone, shogaol, chavicine, piperine, guajacol, isoeugenol, eugenol, xylene, curcumin and mustard oil that show variable degrees of structural and / or pharmacological overlap with capsaicin (Patacchini *et al.* 1990; Takaki *et al.* 1990). Another compound, resiniferatoxin (RTX) **(Figure 2)** has been known since the dawn of the recorded history. RTX occurs naturally in the latex of the Moroccan cactus *Euphorbia resinifera* (Appendino and Szallasi, 1997).

RTX combines the structural features of two classes of natural irritants, phorbol esters and capsaicinoids. Like capsaicin, it contains a 4-hydroxy-3 methoxy benzyl (vanillyl) moiety connected to an ester group which, in the capsaicin molecule, can replace the acylamide group in reversed position without loss of activity (Szolcsanyi and Jancso-Gabor, 1975). This ester group connects the vanillyl moiety to the 20-hydroxyl substituent of the complex diterpene structure. Thus, unlike active phorbol esters, resiniferatoxin lacks a free 20-hydroxyl group which is essential for phorbol ester activity and thus fails to induce the typical phorbol ester effects including tumor promotion, binding to and stimulation of protein kinase C (Szallasi and Blumberg, 1990; Dray *et al.* 1990; Winter *et al* 1990).

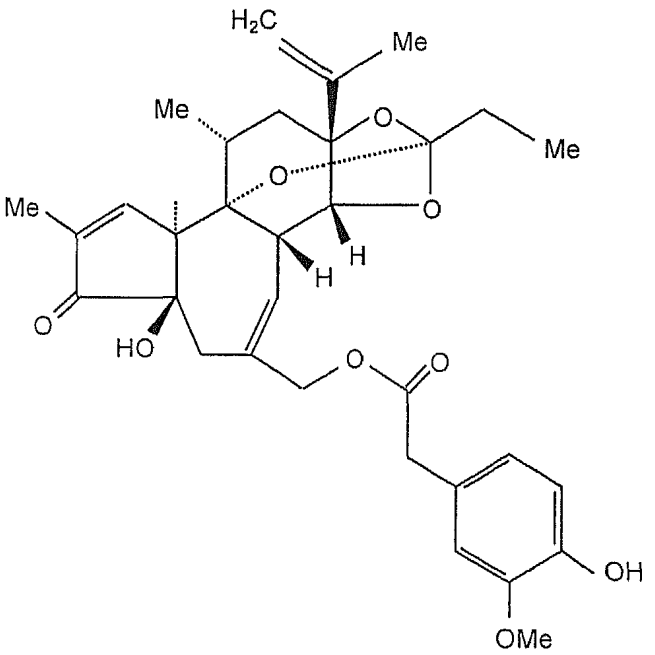
In a series of experiments since 1989, Szallasi *et al* have identified RTX as an ultrapotent capsaicin analog with a unique spectrum of biological activities (Szallasi and Blumberg, 1999). Because capsaicin and RTX analogs share a (homo) vanillyl group as a structural motif essential for bioactivity but differ dramatically in the rest of the molecule, they are collectively termed as vanilloids.

1.3.3 Capsazepine : An antagonist of capsaicin

Capsazepine [{2-2(4-Chlorophenyl) ethyl amino-thio carbonyl}-7,8-dihydroxy-2,3,4,5-tetra hydro-1H 2-benzapine] **(Figure 3)** is structurally related to capsaicin and has been reported to inhibit capsaicin and resiniferatoxin-induced *in vivo* and *in vitro* responses with a competitive mechanism of action (Urban and Dray, 1991; Bevan *et al.* 1992).

Capsazepine was found not to provoke any biological response *per se* (Perkins and Campbell, 1992) and its mechanism of action was, however, clouded by three findings:

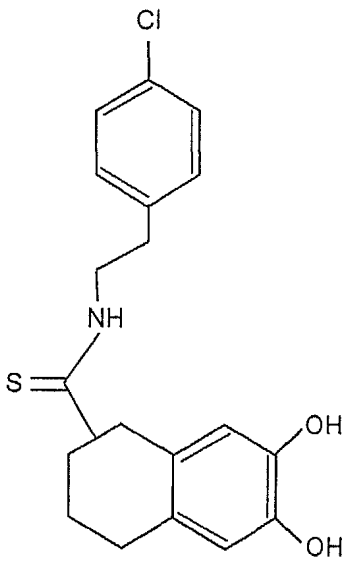
FIGURE 2



RESINIFERATOXIN

Formula : C_{37} $H_{40}O_9$ Formula weight : 628.7

FIGURE 3



CAPSAZEPINE

Formula : $C_{19}H_{21}Cl\ N_2O_2S$ Formula weight : 376.9

(i) Capsazepine was reported to inhibit RTX binding by rat dorsal (DRG) membranes with an unexpectedly poor potency (James *et al.* 1992).

(ii) Its potency to inhibit capsaicin or RTX induced responses was found to vary in the range of 10 to 1000 nM in different preparations (Dray *et al.* 1991).

(iii) Capsazepine was found to block proton-induced "capsaicin-like" biological actions (Lou and Lundberg, 1992).

Capsazepine has therefore been proposed to act on multiple targets (Franco-Cereceda and Lundberg, 1992).

1.4 *MECHANISM OF ACTION OF VANILLOIDS*

Binding of vanilloids to their receptors initiates a complex, and as yet poorly understood cascade of intracellular events, which, for practical purposes, can be divided into three separate phenomena namely : Excitation, Sensitization & Desensitization and Neurotoxicity.

1.4.1 Excitation by vanilloid

On first contact with capsaicin, afferent neurons are invariably stimulated. Administration of capsaicin to the peripheral nerve ending results in depolarization and discharge of action potentials, which in turn evokes burning pain (Stjara *et al.* 1989). This painful sensation is produced by the threshold concentration of 30 nM capsaicin on the eye (Szolcsanyi and Jancso-Gabor, 1975) or blister bast in human skin (Szolcsanyi, 1977), whereas the threshold concentration in the oral cavity and on the tongue of humans is about 0.7 μM

(Sizer and Harris, 1985). When given an as aerosol by nebulising solutions of capsaicin, concentrations of • 2μM produce coughing in humans (Collier and Fuller, 1984).

When capsaicin is administered to the peripheral endings of cutaneous sensory neurons of humans, cat, rabbit and rat, C-fiber polymodal nociceptors (Lang *et al.* 1990), some C-fiber warmth receptors (Kenins, 1982) and some Aδ - fiber polymodal nociceptors are stimulated (Hartung *et al.* 1989).

1.4.2 Sensitization and desensitization by vanilloid

Excitation is followed by sensitization to thermal, mechanical and chemical stimuli, particularly if low doses of capsaicin are administered repeatedly. There is only one report indicating that neuronal sensitivity to capsaicin can increase with repeated applications of the drug to the human tongue; paradoxically, a pause in the stimulation cycle gives rise to desensitization (Green, 1989).

Excitation and sensitization of sensory neurons by vanilloids is followed by a refractory state in which :

(i) neurons do not respond to a subsequent vanilloid challenge, or

(ii) neurons are resistant to various stimuli, ranging from noxious heat to mechanical pressure to endogenous (e.g. histamine and bradykinin) or exogenous (e.g., xylene and mustard oil) algesic-proinflammatory agents.

The refractory state that follows vanilloid treatment is still generally called Desensitization. The duration of desensitization to capsaicin appears to be a matter of few hours to a few days. It is not known whether there is any relationship between desensitization and the presence of capsaicin in the tissue.

1.4.3 Neurotoxicity by vanilloid

It seems that the stimulant and initial neurotoxic effects of capsaicin-related compounds arise from similar mechanisms of action. Two pathways leading to neurotoxicity can be differentiated, one pathway depending on the influx of Ca^{++} and the other involving intracellular accumulation of NaCl. The critical involvement of Ca^{++} in the expression of neurotoxicity is demonstrated by the findings that the neuronal damage caused by capsaicin and its related compound (RTX) is markedly reduced by the removal of extracellular Ca^{++} (Winter, *et al.* 1990) but not by blockade of voltage dependent calcium channel (Marsh *et al.* 1987). These observations have been taken to infer that entry of Ca^{++} into the cell is a priming event for the neurotoxic action of capsaicin.

The neurotoxic effects of capsaicin and its related compound (RTX) on cultured sensory neurons are also reduced when NaCl is replaced by sucrose, but abolished only when, in addition, external Ca^{++} is removed as well, indicating the existence of a second mechanism of neurotoxicity (Winter, 1990).

1.5 BLOOD : COMPOSITION AND PHYSIOLOGICAL FUNCTIONS

Blood is the transport tissue of the body, the store house from which tissues draw their foodstuffs and other substances necessary for their activities and the medium into which tissues discharge their waste products for transportation to the organs of excretion - the kidneys, the intestines, the lungs and the skin. Some of the characteristics of human blood are summarized in **Figure 4.**

The composition of blood is tremendously complex. The composition of the cellular elements is widely different from that of the plasma in which they are suspended in turn, the erythrocytes, leucocytes and platelets differ greatly in composition. Because the erythrocytes represent most of the cellular mass of the blood,they contribute quantitatively most of the cellular constituents. **Table 4** shows the normal ranges or averages of many of the constituents of human blood.

Figure 4

BLOOD

6 - 8 percent of body weight ; 66-78 ml/kg ; 2000-2900 ml/sq.m ;
specific gravity 1.060 av ; viscosity 3.6 - 5.3 ; pH 7.4 av,
range 7.33 - 7.51 ; freezing point 0.55^0C.

PLASMA

50 - 60 percent by volume
Solid 8 - 9 percent
Specific gravity 1.026
Viscosity 1 7 - 2.0
Freezing point 0.55^0C
Total Molal Concentation 0 30
Osmotic pressure at 37^0C 7.6 atm
pH 7.4 av, range 7.33 - 7.51

BLOOD CELLS

40 - 45 percent by volume

Platelets or thrombocytes

200000 - 400000 / cu.mm
Diameter about 2.5 μ

Leucocytes

5000 - 10000 / cu.mm
Composed of mono - cytes, granulocytes and lymphocytes

Erythrocytes

4500000 - 6000000 per cu. mm
Solid 35 percent
Stroma protein 0.5 - 1.0 percent

TABLE - 4

Normal values of blood constituents commonly of clinical importance

Constituent	Normal Range
Hemoglobin, g per 100 ml	
Men	12 - 17
Women	11 - 15
Plasma proteins, g per 100 ml	6.5 - 7.5
Serum proteins, g per 100 ml	6.0 - 6.9
Albumins (serum), g per 100 ml	4.7 - 5.7
Globulins (serum), g. per 100 ml	1.3 - 2.5
A/G ratio	1.2 - 1.8
Fibrinogen (plasma), g per 100 ml	0.2 - 0.4
Glucose, mg per cent (true sugar)	65 - 90
Nonprotein, N, mg per cent	28 - 39
Urea, mg per cent	19 - 33
Urea N, mg per cent	9 - 15
Creatinine, mg per cent	1.2 - 1.5
Uric acid, mg per cent	1 - 3
Cholesterol, total (serum), mg per cent	150 - 250
Cholesterol, free (serum), mg per cent	40 - 70
Cholesterol, esterified (serum), mg per cent	100 - 180
Calcium (serum), mg per cent	4.5 - 5.5
Sodium(serum), mg per cent	135 - 152
Potassium (serum), mg per cent	3.6 - 6.2
Chlorides (serum), as NaCl, mg per cent	98.5 - 104.5
Phosphorus as inorganic phosphate (serum), mg per cent	1.6 - 2.7
CO_2 capacity (serum) at 40mm CO_2,vols per cent	55 - 75
O_2 capacity (exposed to air), vols per cent	16 - 24
Ascorbid acid (plasma), mg per cent	07 - 1.5
Iodine, µg per 100 ml	8 - 15
Bilirubin (plasma), mg per cent	0 2 - 0.8
Amylase, Somogyi units	60 - 180
Lipase (serum), ml N/20 free acid liberated from olive oil, upper normal limit	1.5
Acid phosphatase (serum), units	0.0 - 1.1
Alkaline phosphatase (serum), units	2.2 - 8.6

(West et al., 1976)

1.5.1 Human erythrocyte

The human erythrocyte is one of the most simple cell that has been modified by evolutionary forces into a highly specialized tissue that is mainly responsible for oxygen transport from lungs to the tissues. Erythrocytes **(Figure 5)** are non-nucleated biconcave discs ranging in diameter from 6 - 9 μ, with an average value of 7.5 μ. The thickness of the erythrocyte is 1 μ in the centre and 2 - 2.4 μ towards the periphery. They contain 35 per cent of solids, of which some 31 - 33 per cent is hemoglobin. The specific gravity of erythrocytes averages 1.090. Each erythrocyte contains an estimated 200 - 300 million molecule of hemoglobin.

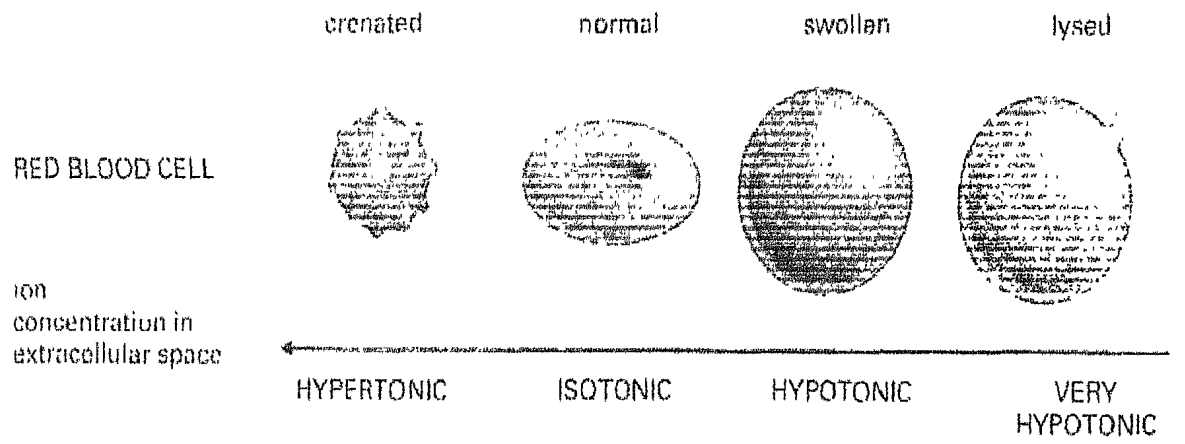
The normal range of erythrocytes per cubic millimeter of blood is shown in **Table 5.**

TABLE - 5

ERYTHROCYTE		
	MEN	WOMEN
Normal Range	4500000 - 6000000 / mm^3	4200,000 - 5400,000 / mm^3
Average	5000000 / mm^3	4500,000 / mm^3

The numbers of erythrocytes may be enormously increased in polycythemia and decreased in some of the anemias. It has been estimated that the adult human body contains around 10^{12} erythrocytes which carry about 950 g of hemoglobin.

Figure 5



HUMAN ERYTHROCYTES

1.5.2 Red cell membrane

Red cell components are surrounded by a limiting membrane that keeps it separated from the extracellular environment. Its normal functioning is indispensable for the survival of red blood cells in circulation by virtue of its role in determining the red cell shape, size and its deformability characteristics. Any disorder in the normal functioning of cell membrane properties, due to changes in its structure and / or composition or due to change in cell shape, leads to diminished red cell survival.

The erythrocyte membrane is composed of a lipid bilayer that contains several integral proteins and a protein lattice that underlies the bilayer and is associated with it through protein-protein and protein-lipid interactions. This protein lattice is often referred to as the membrane skeleton, it consists of spectrin, actin, tropomyosin and proteins 4.1 and 4.9. The evidence accumulated to date suggests that membrane deformability and stability are predominantly determined by these membrane skeletal proteins (Chasis and Shohet, 1987).

The erythrocyte membrane is readily permeable to H_2O, CO_2, urea, glucose and creatine and ions HCO_3^-, Cl^- and OH^-. Most of the erythrocyte cations are K^+, but a small amount of Na^+ is also present. Most of the Ca^{++} is in the plasma and Mg^{++} in the cells. The distribution of smaller uncharged molecules between erythrocytes and plasma shows great variations. Some substances such as glucose and urea are distributed between plasma and cells according to their water content, whereas other substances, such as glutathione appears to be localized entirely within the cells.

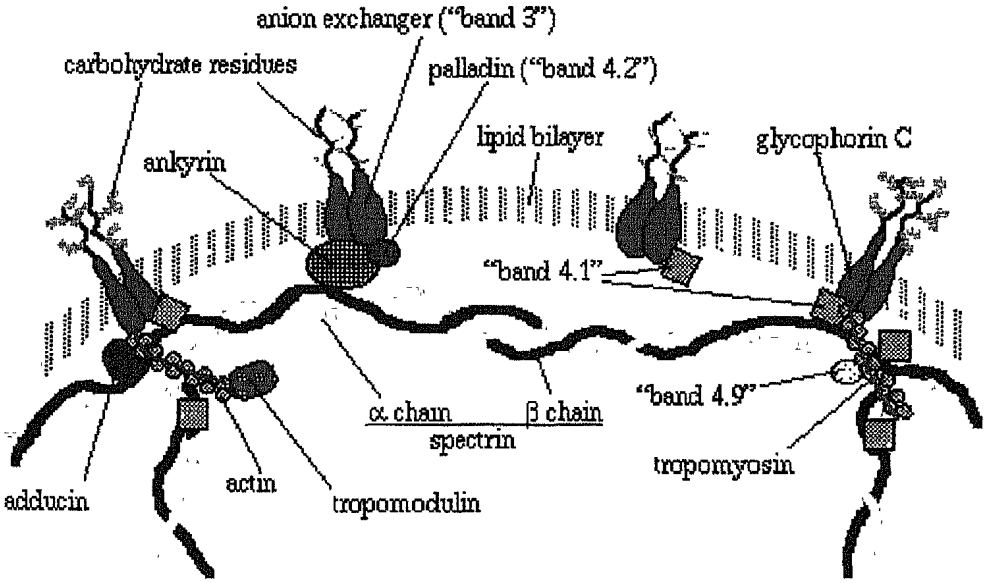
1.5.2.1 Structure of the RBC plasma membrane

The cells are separated from the surrounding medium by a limiting boundary, which approximates a two dimensional planar structure around a cell in contact with its environment, distinct from that of the cell. The plasma membrane acquired its characteristic shape due to the presence of attachments that reaches into the cell and forms cytoskeleton. A notable feature of living cells is that of the functioning of transport systems within the plasma membrane that play a role in changing the environment on either side of the membrane.

Among the most remarkable features of the living organism is the coordination of its cellular metabolism. The contribution of biological membranes is to separate compartments from one another in which a particular biochemical reaction can take place unhindered by other biochemical reactions of other unnecessary external and/or internal influences.

Biomembranes are composed mainly of proteins and lipids with a small amount of carbohydrates present in association with proteins as glycoprotein and/or lipids as glycolipids. Various molecular components of membrane are organised in a unique manner to give rise to a functional membrane. Thermodynamic stability of the membrane demands that lipid-protein interaction within the membrane must maximize hydrophobic interaction between non-polar parts and hydrophilic interaction between polar parts of various molecules. The potential hydrophobic groups are generally amino acid residues of membrane proteins and non-polar parts of lipid. The hydrophilic group constitutes the ionic, zwitter ionic and polar groups of lipid, protein, protein and carbohydrate components of the membrane **(Figure 6).**

Figure 6

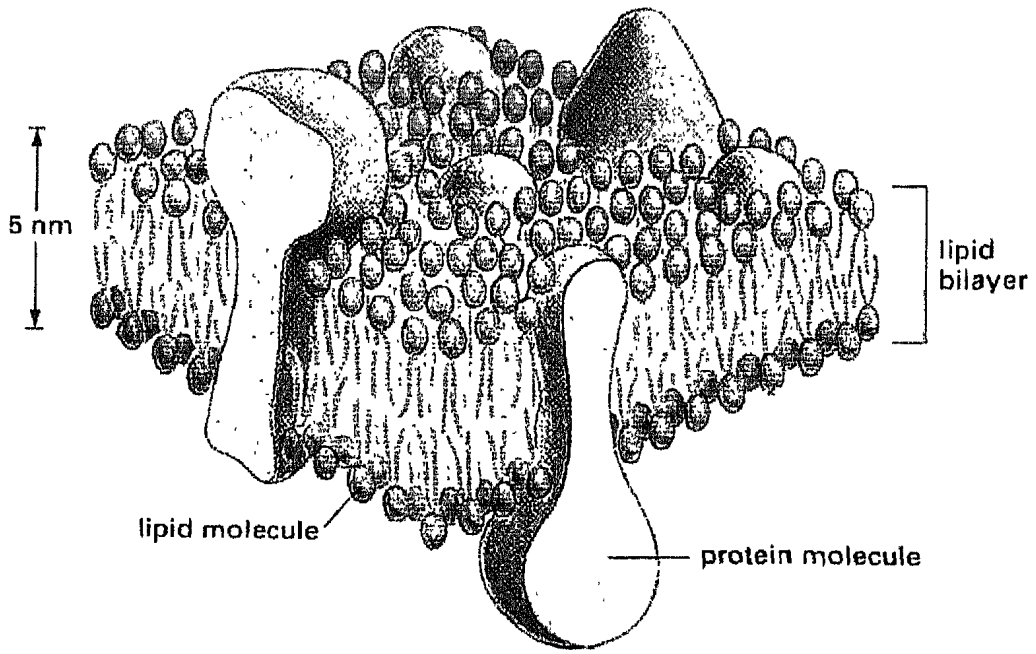


STRUCTURE OF THE RED CELL MEMBRANE

Different authors have proposed models of membrane structure and molecular organisation. Gorter and Grendel (1925) proposed that basic structure of cell membrane is that of a lipid bilayer. Davson and Danielli (1943) elaborated further and proposed that cell membranes are composed of lipid bilayer structure where the phospholipids are arranged in an orderly fashion with hydrophilic polar head groups towards the two surfaces facing the hydrophilic environment on outer and inner side of the membrane. They suggested that the membrane proteins were present on the surface of lipid bilayer attached to hydrophilic polar group of phospholipids. The membrane model proposed by Davson and Danielli was further modified by Robertson (1959) who suggested that surface protein were present as a monolayer. But the 'Fluid Mosaic Model' proposed by Singer and Nicholson (1972) is most accepted as it fulfills the criteria for the functional biological membrane. It takes into account both the variation in molecular size of protein and various classes of lipids present in varying proportion in the membranes of different species. This model was able to explain the concept of fluid and dynamic nature of the membrane, interpreting that the matrix of membrane was constituted of phospholipids in fluid state arranged in the form of planar bilayer organisation and protein molecules are either floating, submerged or transversing through the phospholipic bilayer forming the mosaic of the bilayer **(Figure 7)**.

Fluid Mosaic Model distinguishes between peripheral (extrinsic) and integral (intrinsic) proteins of the membrane. Extrinsic proteins are those, which are held by membrane on generally their surface by weak non-covalent forces and are not strongly associated by lipids. These require only mild treatment for their dissociation from the membrane and in their dissociated state are readily soluble

Figure 7



FLUID MOSAIC MODEL OF PLASMA MEMBRANE
(Singer and Nicholson, 1972)

in neutral aqueous buffers. Intrinsic proteins are usually strongly associated with lipids even in their isolated state. These require drastic treatment for their separation from membrane and are usually insoluble in neutral aqueous buffers. Intrinsic proteins undergo translational diffusion within the membrane, depending on the effective viscosity of lipids unless tied down by some specific forces. Integral proteins because of amphiphatic structure maintain their orientation and the degree of intercalation within the membrane while undergoing translational diffusion in the plane of the membrane.

1.5.2.2 Composition of red cell membrane

A large amount of study has been done by biochemists in the past few decades on the composition, structure and specific function of various components of red blood cell membrane. As any other plasma membrane, human red blood cell membrane is also composed mainly of proteins, lipids and carbohydrates in varying proportions. Chemical composition of red cell ghost, which is accepted as that of red cell membrane, is given in **Table 6** (Rosenberg and Guidotti, 1968) and the distribution of proteins, lipids and carbohydrates in the peripheral and integral parts of membrane is given in **Table 7** (Fairbanks, 1980).

TABLE - 6

Chemical composition of red cell membrane as given by Rosenberg and Guidotti (1968).

	Amount in per cent
Protein	42.9
Total Lipids	43.6
Phospholipids	32.5
Cholesterol	11.1
Carbohydrates	7.2
Neutral Sugars	4.0
Hexosamines	2.0
Sialic acid	1.2

TABLE - 7

Chemical composition of red cell membrane as given by Fairbanks (1980).

	Amount in percent	
Protein	52	45% of total protein is Integral protein
		55% of total protein is Peripheral protein
Lipids	40	33% of total lipids is Cholestrol
		67% of total lipids is phospholipids
Carbohydrate	8	93% of conjugated carbohydrate as Glycoprotein
		7% of conjugated carbohydrate as Glycolipids.

1.5.3.2 Red cell membrane proteins

The human erythrocyte membrane proteins constitute approximately 50% of the dry weight of red cell membrane. The major protein component comprises about 60% of the total membrane protein: including spectrin (bands 1 and 2), ankyrin (band 2 1), bands 2.2, 2.3, 2.6, 4.1, actin (bands 5) and a proportion of the protein designated as bands 3, 4.2, 4.9 and 7.3

Spectrin, a major protein of the erythrocyte membrane skeleton, is composed of two non-identical subunits 'a' and 'b' (bands 1 and 2). It is a peripheral protein which gives framework to the erythrocyte membrane (Branton *et al.* 1981).

Ankyrin (bands 2.1) is a monomeric globular acylated phosphoprotein, links spectrin to the cytoplasmic domain of band 3. The erythrocyte membrane contains two predominant integral proteins, band 3 and glycophorin A, that span the phospholipid bilayer with distinct domains expressed on outer and cytoplasmic surfaces (Statfenbiel and Lazarides, 1986).

Band 4.1, a peripheral protein including two polypeptides, is reported to be involved in the interaction of spectrin with actin. Like ankyrin, band 4.1 also binds to the cytoplasmic segment of transmembrane glycoprotein. (Anderson and Marchesi, 1985). Band 4.2 is associated with the cytoplasmic domain of band 3. Actin is reported to be the component of membrane skeleton. Moreover, an acidic cytoplasmic protein having cylindrical shape i.e. cylindrin has also been reported (Harris, 1988).

1.5.2.3 Red cell membrane lipids

Lipids the other very important constituents of the membrane, comprise approximately 40% of the total red cell membrane by weight. The main lipid components are phospholipids, cholesterol and glycosphingolipids. The human erythrocyte membrane contains 70% phospholipids, residual 30% being made up of cholesterol with small proportions of glycolipids and free fatty acid (FFA). It is universally accepted that the various classes of phospholipids are organised asymmetrically between the two leaflets of the lipid bilayer. This may play an important role in maintaining the functional integrity of mature red cells, which cannot synthesize their own phospholipids (Nelson, 1972).

Phosphatidylcholine (PC), the most prevalent membrane phospholipid is predominantly localised on the outer leaflet of the membrane whereas phosphatidylethanolamine (PE) and phosphatidylserine (PS) are on the inner leaflet of the membrane. This asymmetrical distribution of phosphilipid is probably due to specific interaction between its various classes.

Cholesterol, one of the major components of the lipid in the red cell membrane, has been found to exchange freely between the lipid bilayer and is in equilibrium with the free (non-esterified) cholesterol attached to the plasma lipoproteins. Cholesterol associated with phospholipids may play a key role in modulating the fluidity and permeability characteristics of the membrane bilayer and consequently the membrane function (Bloch, 1983).

1.5.2.4 Red cell membrane carbohydrates

Carbohydrates are present on exterior surface of the membrane in the form of oligosaccharide moieties as conjugated glycolipids and glycoproteins,

are mostly responsible for surface charge and surface determinant properties. It is well known that erythrocytes have bulk of negative charge on their outer surface, which helps them to avoid their self-aggregation in plasma (Tanner, 1979).

The glycoproteins located at the external surface of the cell membrane play an important biological role as red cell surface antigen and receptor sites. These glycoproteins are also involved in several phenomena such as contact inhibition, cell-cell interaction and neoplastic transformation. Glycophorin A, the basic subunit of dimer, represents, approximately 75% of the total sialoglycoprotein of the erythrocyte membrane, and carries MN antigens. Glycophorin B carries Ss blood group activity **(Table 8)**.

TABLE - 8

Protein	Units of Organisation	No.per Ghost	Proposed function
Spectrin	$(1,2)_2$	110,000	Skeleton
Ankyrin, Syndein	2.1+2.2	100,000	Skeleton anchor
Band 3	$(3)_2$	470,000	Anion transport
	4.1	180,000	Skeleton anchor
	$(4.2)_4$	60,000	?
Actin	$(5)_4$	90,000	Skeleton
G-3-PD	$(6)_4$	135,000	Glycolysis
	7	403,000	?
Glycophorin	$(GP)_2$	250,000	MN antigens

(Fairbanks, 1980).

1.5.2.5 Membrane fluidity

Fluidity (ϕ) is the property of a liquid that describes its ease of movement. It is represented by the following equation

$$\text{fluidity} = \phi - 1/\eta = 1/\text{Viscosity}.$$

In 1966, Chapman *et al.* postulated a fluidity concept for biomembranes that proposed "the particular distribution of fatty acyl residues which occurs with a particular biomembrane is present so as to provide the appropriate membrane fluidity for a particular environmental temperature to match the required diffusion rate or rate of metabolic processes required for the tissues." It was also suggested that "there appear to be biosynthetic feedback mechanisms by which a cell will attempt to retain a constant membrane fluidity and example were given of poikilothermic organisms that altered their membrane fluidity to match different environmental temperatures. The molecular details underlying the membrane fluidity concept are as under:

1. The lipid chains show flexing and twisting of the methylene (i.e. CH_2) groups and a marked increase of rotational isomers.

2. The oscillations and rotational disorder of the methylene groups are most marked at the methyl end of the lipid chains (Chapman and Salsbury, 1966).

3. The polar groups of the lecithin molecules exhibit a marked increase in mobility (Veksli *et al.* 1969).

4. Lipid self-diffusion occurs when sufficient water is present to weaken any ionic linkages between the polar groups (Penkett *et al.* 1968).

5. The transition temperature from ordered to fluid "melted" chains is related to the chain length and degree of unsaturation of the lipid (Chapman *et al.* 1967).

6. The fluidity of the biomembrane can be modulated by molecules which penetrate the lipid bilayer such as Cholesterol (Chapman and Penkett, 1966; Ladbrooke *et al.* 1968).

7. The fluidity of the biomembranes is affected by hydrogenation (Chapman and Quim, 1976) polymerization (Johnston *et al.* 1980) Photooxidation (Restall *et al.* 1981) and irradiation with UV radiation (Leaver *et al.* 1982).

1.5.3 Red blood cell metabolism

1.5.3.1 Intracellular metabolite and their importance

A mature red blood cell has no nucleus or mitochondria. It depends on glycolysis for energy (to maintain cationic pumps, membrane integrity etc.) They lose their capacity to synthesze amino acids, new proteins and fatty acids along with oxidative phosphorylation. Due to loss of biosynthetic capability these cells are unable to replace enzymes, repair their membrane and utilize oxygen as a source of energy. Red blood cells maintain their physiological state through the supply of energy in the form of ATP formed exclusively by the breakdown of glucose and its utilization mainly by Embden-Meyerhof pathway. In addition, red blood cells also have the capacity for NAD^+ / $NADP^+$ reduction and glutathione synthesis (GSH) along with formation of 2,3 DPG. The formation of reduced glutathione enables red cell to protect sulfhydryl (-SH group of proteins against

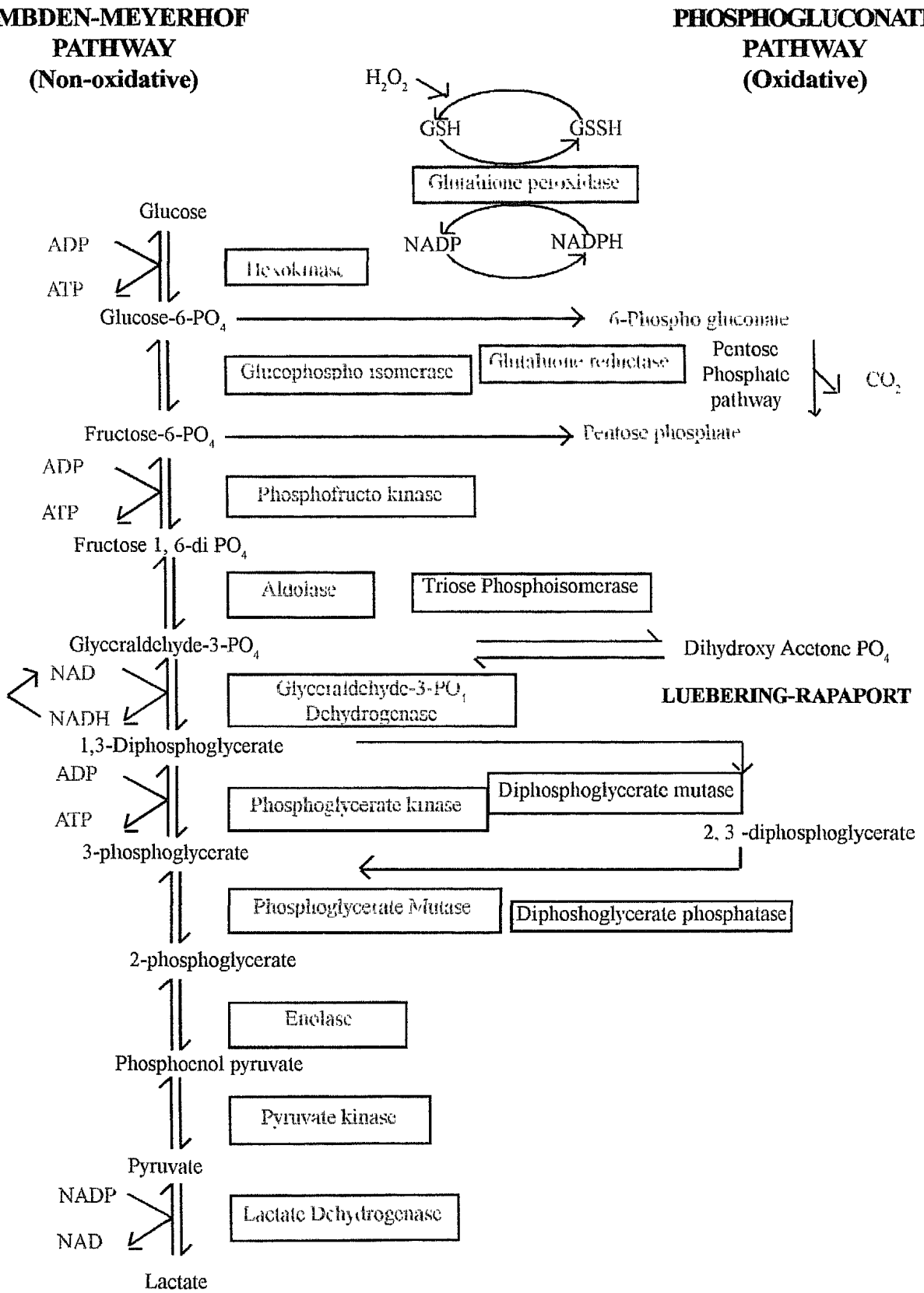
oxidation and to trap metallic ions by forming mercaptides.

Other pathways such as phosphogluconate pathways (hexose monophosphate shunt), methemoglobin reductase pathway and Luebering Rapoport pathway are related to EMP. **(Figure 8).** It is necessary for all these pathways to function adequately if the erythrocytes are to survive and transport oxygen normally.

1.5.3.2 ATP : The prime source of energy

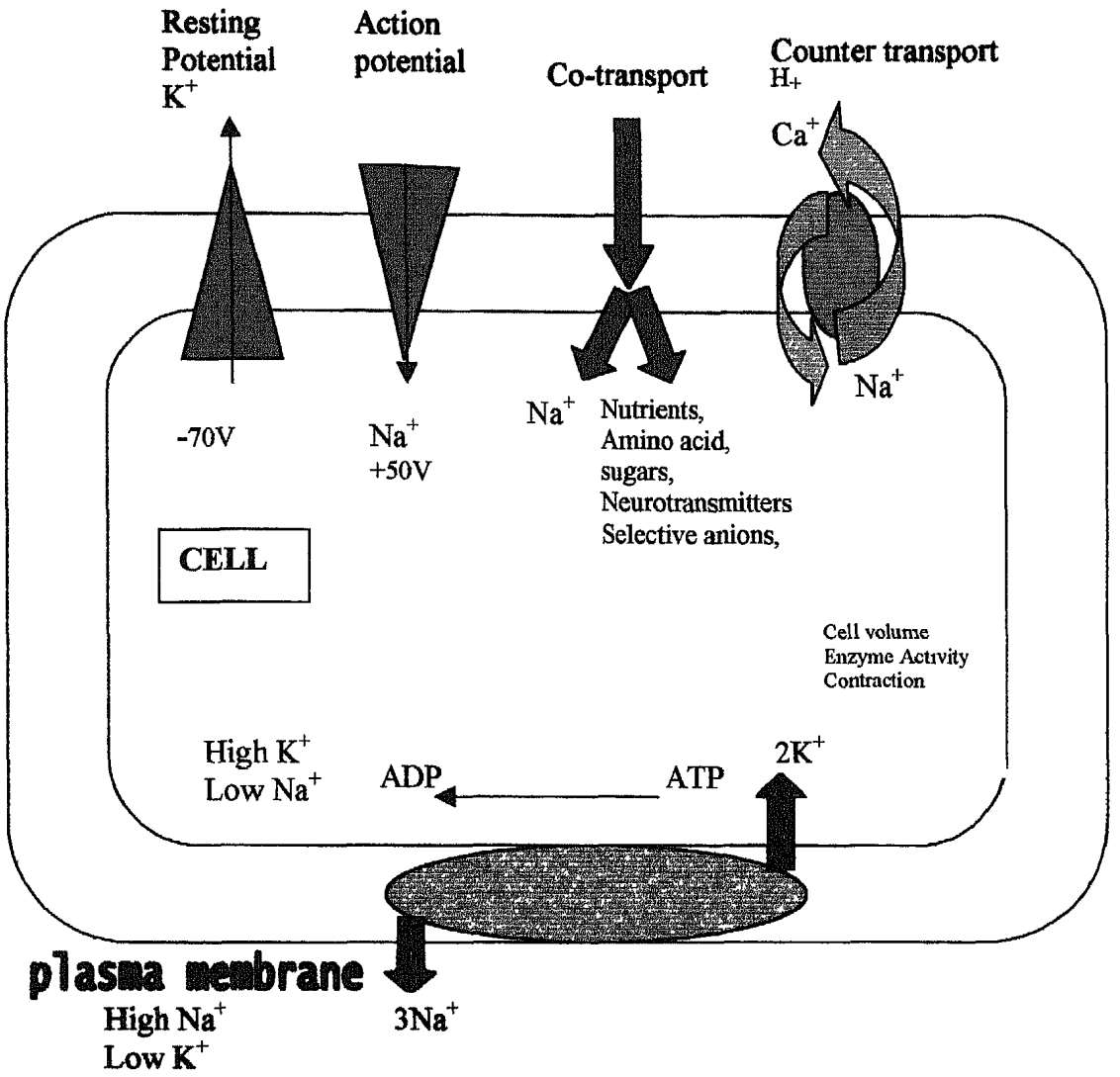
ATP the prime source of the energy of red cells, plays an important role associated with cellular activity of red cells. These include :

1. Phosphorylation of various metabolites present in RBC particularly glucose to initiate the process of glycolysis.
2. The transport of Na^+ out and K^+ into the cell by Na^+/K^+-ATPase (Sodium pump) to maintain the ionic balance inside the cell. **(Figure 9).**
3. Transport of calcium through Ca^{++}-ATPase (Calcium pump)
4. To maintain red cell biconcave shape and flexibility. A relationship has been reported between the red cell shape, its deformability and the amount of available ATP.
5. Regulation of EMP and its connected pathways by allosteric modulation of Hexokinase, Phosphofructokinase and Pyruvate kinase enzymes.
6. As a substitute for 2,3 DPG in modulating the position of oxygen dissociation curve.



RED BLOOD CELL METABOLIC PATHWAYS

Figure 9



SCHEMATIC ILLUSTRATION OF MEMBRANE TRANSPORT AND CELLULAR FUNCTIONS

1.5.3.3 Oxidised (GSSG) and reduced (GSH) glutathione

Glutathione is a tripeptide of glycine, cysteine and glutamate. The biosynthesis of GSH by mature red cells enables them to maintain a high intracellular concentration (approx. 70 mg / dl or 2.2 mM). The cell membrane is impermeable to GSH, also the oxidized form (GSSG) cannot enter red cells but at high concentrations can leave them in an energy dependent reaction. The GSSG concentration is usually low, about 10% of the GSH + GSSG level, and it is not clear whether GSSG leakage is a normal occurrence.

GSH has a half life of about three days. The major role of GSH in red cells is to protect protein - SH groups against oxidation and to trap unwanted metallic ions by forming mercaptides. Apart from this, GSH also helps in scavenging free radicals. GSH participates in three other activities.

1. Glyoxalase reaction for the conversion of methyl glyoxal to lactate in which GSH acts as a co-factor.
2. As a prosthetic group bound to glyceraldehyde -3-phosphate dehydrogenase (GAPD).
3. Detoxication of certain foreign compounds to yield mercapturic acid

1.5.4 Enzymes involved in maintaining redox state of red blood cells

1.5.4.1 Glutathione reductase (GR)

This enzyme catalyses the reduction of GSSG to 2 GSH using NADPH or NADH as hydrogen donor:

$$GSSG + NADPH + H^+ \rightarrow 2\ GSH + NADP^+$$

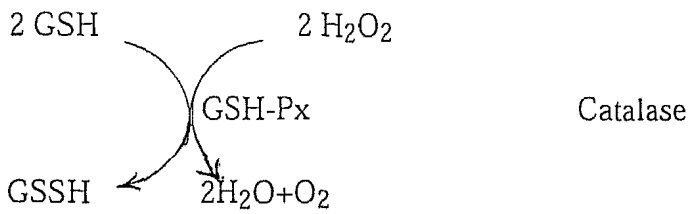
or $$GSSG + NADH + H^+ \rightarrow 2\ GSH + NAD^+$$

The enzyme is inhibited by chromate and by 2,5 dinitrobenzoic acid.

GR contains FAD as a prosthetic group and it is possible to increase red cell GR activity *in vitro* by the addition of FAD to hemolysates, or *in vivo* by ingestion of 5 mg daily of riboflavin GSSG is not a unique substrate of GR which will reduce dihydrolipoic acid and also the mixed disulphides formed between GSH and protein -SH groups; the latter reaction was demonstrated with mixed disulphides of hemoglobin and was thought to be of significance when red cells are assaulted by oxidative agents.

1.5.4.2 Glutathione peroxidase (GSH - Px)

Decomposition of the highly toxic H_2O_2 is carried out in two reactions catalyzed by catalase and by GSH-Px.



The relative contribution made by the 2 enzymes depends upon the generation of H_2O_2; at low rates GSH-Px is the major route while at higher rates catalase becomes more important. GSH-Px is highly specific for GSH.

1.5.4.3 Catalase

Catalase functions as a peroxidase and catalyze reactions of the type :

$$AH_2 + H_2O_2 \rightarrow A + 2\,H_2O$$

in which A may be a phenol, an alcohol or ascorbate. The peroxidase reaction

occurs in more than one stage starting with the attachment of H_2O_2 to the enzyme to form complex I.

$$\text{Protein-Fe}^{3+}\text{-OH} + H_2O_2 \rightarrow \text{Protein -Fe}^{3+}\text{ -O-OH+ } H_2O$$

(Complex I)

Complex I is green and sensitive to oxidants which may produce an inactive complex II which is red.

$$\text{Protein-Fe}^{3+}\text{-O-OH} + AH_2 \rightarrow \text{Protein-Fe}^{3+}\text{ - OH+}H_2O + A$$

(Complex II)

Small amounts of Complex II are present in red cells and its reactivation by NADPH links catalase activity with the pentose phosphate pathway so that routes for H_2O_2 breakdown can be linked to glycolysis.

The peroxidatic activity of catalase is due to its prosthetic group, a porphyrin ring containing Fe^{3+} iron. Hemoglobin also displays peroxidative activity when the iron is in the ferric state to give methaemoglobin.

2. *REVIEW OF LITERATURE*

The last decade (1990-2000) saw an exponential increase in publications related to capsaicin and its application as a therapeutic agent. A simple Medline search on capsaicin yields over 3000 publications since 1990.

2.1 CAPSAICIN : A 'HOT' TOOL

The turning point in the history of capsaicin was 1949 when N. Jancso realised that capsaicin is unique among the naturally occuring irritant agents in that the initial stimulation by capsaicin is followed by a rapidly developing refractory state that he termed desensitization (Jancso and Jancso-Gabor, 1949; Jancso, 1955).

Jancso was first to postulate that capsaicin acts on a "pain receptor" to produce its neural effects. In 1975, Szolcsanyi and Jancso-Gabor described the first receptor model. Consequently, over the past three decades several attempts have been made to demonstrate the actual existence of capsaicin receptors by using radiolabelled dihydrocapsaicin (Szebeni *et al.* 1978; Millev *et al.* 1982) or capsaicin like photo affinity probes (James *et al.* 1988).

2.2 VANILLOID RECEPTOR

Capsaicin and its related compounds (capsazepine and resiniferatoxin) collectively referred to as vanilloids differ dramatically in the rest of the molecule but share a homovanillyl group essential for biological activity. In keeping with this terminology, the receptor at which these ligands interact is referred to as the vanilloid receptor (VR) (Szallasi, 1994).

In 1990 Szallasi *et al.* were the first to demonstrate high affinity; saturable specific binding sites for RTX shared by capsaicin (Szallasi and Blumberg, 1990a). It is generally accepted that only mammalian species respond to capsaicin in a specific manner, no specific RTX binding could be detected in the sensory ganglia of reptiles (alligators) or birds (chicken). Specific binding of RTX is fully displaced by capsaicin but it is not inhibited at all by biologically inactive capsaicin and RTX congeners ; neither it is inhibited by the functional vanilloid receptor antagonist Ruthenium red, an inorganic dye which is thought to interfere directly with the cation transport via the vanilloid receptor linked conductance (Amann and Maggi, 1991).

Further evidence for the existence of a vanilloid receptor comes from the development of a selective antagonist of RTX and capsaicin actions, named capsazepine (Urban and Dray, 1991; Bevan *et al.* 1992; Walpole and Wrigglesworth, 1993). Capsazepine reversibly inhibits vanilloid induced biological responses with a competitive mechanism (Bevan *et al.* 1992). Interestingly, capsazepine also prevents the proton induced activation of sensory nerves (Fox *et al.* 1995).

David Julius's group at the University of California in San Francisco has successfully cloned the receptor for capsaicin. They named the receptor *vanilloid receptor sub type 1* (VR 1). The cloned VR 1 cDNA contains 2514 nucleotides that encodes a protein of 838 amino acids with a molecular mass of 95 K Da.

2.2.1 *Heat and pH activate VR 1*

An exciting aspect of the cloning of VR1 is the finding that both noxious heat (a rapid increase in temperature from 22°C to 48°C) and low pH are

able to activate the capsaicin - gated channel (Tominaga *et al.* 1998). Infact, this may be the very reason why capsaicin is "hot tasting" to humans. Thus VR1 can be viewed as an integrator of painful chemical and physical stimuli. Probably, it is heat only that has the power to open VR1, capsaicin and low pH serve to reduce the heat threshold of the receptor. Consequently, even room temperature is able to open VR1 under mildly acidic conditions. A reduction from 7.6 to 6.3 in the pH of the bath solution does not open VR1 expressed in oocytes; nonetheless, it results in a 5-fold increase in the amplitude of the current evoked by 300 nM of capsaicin (Caterina *et al.* 1997). This is consistent with the earlier observations that

(i) low pH potentiated responses to low concentrations of capsaicin in rats (Kress *et al.* 1996), rabbit (Martenson *et al.* 1994), or human (Baumann *et al.* 1996) sensory neurons in culture and

(ii) low pH evoked a current in cultured trigeminal neurons that could be prevented by the VR antagonist capsazepine (Liu and Simon, 1994).

A further reduction in the pH to 5.0, however, evokes a current through VR1. Moreover, hydrogen ions can increase the response of VR1 to noxious heat (Caterina *et al.* 1997). Protons also inhibit RTX binding to DRG neurons or spinal cord membranes (Szallasi *et al.* 1995). It must be mentioned, that VR1 represents only one target - and not "the target" - for noxious heat or acids in sensory neurons.

2.3 BIOLOGICAL ACTION OF VANILLOIDS (CAPSAICIN)

2.3.1 Pungency

Vanilloid ligands show striking differences in biological actions. Some of

these differences can be explained at the level of a single receptor. For instance, the archaetypal vanilloid, capsaicin, is both pungent and desensitizing. Piperine (*Piper* sp.) is pungent (Szolcsanyi, 1982) but does not desensitize (Liu and Simon, 1996). In the case of olvanil (*Brassiea* sp.) the pattern is the opposite : it is non- pungent (Dray *et al.* 1990) but desensitizing (Liu *et al.* 1997). These differences in biology are likely to reflect differences in kinetic, affinity, and sensitivity to antagonists (Liu *et al.* 1998).

2.3.2 Action of vanilloids on sensory neurons

Upon activation, vanilloid-sensitive neurons release a variety of pro-inflammatory mediators (notably, tachykinins as well as calcitonin gene-related peptide) which in turn, initiate a cascade of inflammatory events collectively referred to as neurogenic inflammation. These evoke a number of protective responses (e.g. coughing) and transmit nociceptive information to the central nervous system (Buck and Burks, 1986: Maggi and Meli, 1988; Holzer, 1988; Maggi, 1991; Lundberg, 1993). Among the known activators of vanilloid-sensitive nerves, protons are suspect "endogenous vanilloids". Not only are proton-induced ion currects strikingly similar to those induced by vanilloids (Bevan and Heats, 1991; Petersen and La Motte, 1993; Liu and Simon, 1994) but proton-induced activation of sensory nerve is also inhibited by the competitive vanilloid antagonist capsazepine (Lou and Lundberg, 1992; Franco-Cereceda and Lundberg, 1992; Santicioli *et al.* 1993; Fox *et al.* 1995).

Neurogenic inflammation is thought to contribute to the symptoms of various allergic/hyperreactive disorders (Maggi and Meli, 1988; Maggi, 1991; Lundberg, 1993). Moreover, from Crohn's disease (Mantyh *et al.* 1988) to

bronchial asthma (Peters *et al.* 1992), a number of disease states are known to be associated with dramatically increased expression of tachykinin receptors. Thus, agents which can interfere with the synthesis and/or proinflammatory neuropeptides from vanilloid-sensitive neurons have a clear therapeutic potential. (Cater, 1991; Maggi, 1992; Campbell *et al.* 1993; Szallasi and Blumberg, 1993).

The mechanism of action of vanilloids suggests that the vanilloid receptor is either a ligand gated nonspecific cation channel *per se* or is at least closely coupled to one (Bevan and Szolcsanyi, 1990; Dray, 1992; Bevan and Docherty, 1993; James *et al.* 1993). The vanilloid receptor / channel complex is expressed in the presence of nerve growth factor only (Winter *et al.* 1993). The opening of this cation conductance leads to impulse generation (afferent function) and to the above described release of neuromediators (efferent function). Excitation by vanilloids is followed by a refractory state which can be either reversible (traditionally termed desensitization) or irreversible most likely reflecting gross neurtoxicity (Holzer, 1991; Dray, 1992). Desensitization is presumably a very complex process in which stages may be distinguished (Szolcsanyi, 1993); these putative stages are likely to involve distinct mechanisms (Szolcsanyi, 1993). Interestingly, whereas the functional desensitization is entirely dependent on the presence of extracellular calcium (Santicioli *et al.* 1987). Pharmacological desensitization at least to a certain degree, may also be achieved in the absence of extracellular calcium (Holzer, 1991). It has been suggested that functional desensitization is due to dephosphorylation by calcium activated phosphatases, such as calcineurin, of the vanilloid receptor itself as well as other receptors, ion channels or enzymes (Yeats *et al.* 1992; Bevan and Docherty, 1993). The

underlying mechanisms of pharmacological desensitization are essentially unknown. Vanilloid induced neurotoxicity is thought to be due to a combination of intracellular calcium accumulation and the osmotic stress associated with cation influx (Bevan *et al.* 1987; Bevan and Szolsanyi, 1990; Holzer, 1991).

2.3.3 Species related differences in vanilloid actions

It has long been known that vanilloids show striking species-related differences in biological actions (Holzer, 1991). In principle, these differences reflect :

(i) Species -related differences in VR expression.

(ii) Species - related differences in neurotransmitter expression in vanilloid-sensitive neurons.

(iii) Species - related differences in the expression of receptors for these neurotransmitters (Glinsukon *et al.* 1980).

2.3.3.1 Differences in mammalian species

Among mammalian species, rabbits are distinguished by their marginal sensitivity to capsaicin (Tervo, 1981). Hamsters are noted for their resistance to capsaicin (Maggi *et al.* 1987), which is in accord with the lack of detectable RTX binding sites in their peripheral tissues. Although guinea pigs are very sensitive to vanilloids (Bucks and Burks, 1986), neither the affinity nor the density of RTX binding sites in this species exceeds the parameters determined in the rats (Szallasi *et al.* 1995). However, unlike in guinea pigs, in humans the bronchoconstrictor action of capsaicin is apparently not mediated by tachykinins (Ellis *et al.* 1997). The only known response that capsaicin reproducibly provokes in healthy human airways is cough (Karlsson, 1996).

The affinity of specific RTX binding sites and their density seem to be fairly similar in human (Acs *et al.* 1994), monkey and porcine spinal cord (Szallasi *et al.* 1994).

2.3.3.2 Differences in non-mammalian species

In contrast to mammals, which in general are sensitive to the irritant and long term inhibitory effects of capsaicin on sensory neurons, non-mammalian species appear to be only poorly sensitive to the drug (Daniel *et al.* 1987).

Administration of capsaicin in pigeons and other birds, at concentrations of 30 mM or doses upto 600 mg / kg, either fails to provoke pain or is only weakly active (Sann *et al.* 1987). Capsaicin (10 μ M) is unable to evoke a detectable release of substance P from these neurons. No change in chemonociception have been noted. Thermo-regulation has been examined in some avian species and was shown not to be affeced by the capsaicin (Geisthovel *et al.* 1986).

2.3.4 Action of vanilloids on non-sensory neurons and non-neuronal cells

The acute actions of capsaicin, however, are not restricted to neurons, and there are a number of reports of capsaicin influencing non-neural systems. These cell non-selective effects of capsaicin and capsaicin congeners include: inhibition of cardiac muscle excitability (Franco-Cereceda and Lundberg, 1988), inhibition of visceral smooth muscle activity (Takaki, 1990) and contraction of vascular smooth muscle (Holzer *et al.* 1990), contradictory data suggest that capsaicin and related substances might either enhance (Moritoki *et al.* 1990) or inhibit (Flynn *et*

al. 1986) the formation of prostanoids in vascular tissue of rabbits and humans, respectively, whereas no effect on prostanoid formation is observed in rat gastric mucosa and other tissues (Brand *et al.* 1990; Holzer, 1991).

In addition, capsaicin and its congeners have been reported to inhibit platelet aggregation (Wang *et al.* 1985) and to influence a variety of enzymatic activities (Srinivasan and Satyanarayana, 1989; Yagi, 1990) and other cell and tissue functions (Matucci - Cerinic *et al.* 1990).

Importantly, many of the cell-non-selective effects of capsaicin are produced by, or were studied with, doses of the drug far in excess of those necessary to stimulate thin afferent neurons. In addition, capsaicin non-selective actions differ from its stimulant action on sensory neurons in that they are sustained, do not undergo desensitization, and are easily reproducible on reapplication of capsaicin (Edvinsson *et al.* 1990).

Long time exposure to capsaicin (both dietary and medicinal) may be mutagenic, may promote tumour formation or may act as a complete carcinogen (Surh and Lee, 1995). It is important to note that it is not capsaicin but it's liver metabolite that may be hazardous.

Laboratory studies indicate that chilli peppers may induce tumour formation in the gastro-intestinal tract of rodents (Agrawal *et al.* 1986), whereas studies in Mexico (Lopez-Carillo *et al.* 1994) and India (Notani and Jayant, 1987) found a significant co-relation between chilli pepper consumption and risk of gastric cancer. The Italian study reached the opposite conclusion : eating hot pepper on a regular basis protected against carcinoma of the stomach (Bulatti *et al.* 1989). Further studies are needed to confirm or rule out the hazards of hot pepper consumption.

An interesting observation is that capsaicin can inhibit the growth of a number of transformed cell lines (Moore *et al.* 1995). It is believed that the transformed cells express an unusual NADH oxidase isoform that is absent in normal cells (Yantin *et al.* 1998). This putative enzyme is the suggested target for capsaicin. Capsaicin also inhibits the plasma membrane electron transport system and induces apoptosis in transformed cells (Macho *et al.* 1999). The finding suggest that the plasma membrane electron transport system may be an interesting target to design anti-tumoural and anti-inflammatory drugs.

2.4 CURRENT USES AND FUTURE PERSPECTIVES OF VANILLOIDS

In principle, all the three characteristic actions of vanilloids (excitation, desensitization and neurotoxicity) may have therapeutic value (Szallasi and Blumberg, 1993). Stimulation (counter - irritation) and desensitization are already in use in clinical practise.

Capsaicin is classified by the FDA as a counter irritant, a substance which may be deliberately applied to the skin to releive irritation and pain. It is a standard ingredient in a variety of over the counter drugs (e.g. Heet, Stimurub, Capsoderma) used world wide to relieve muscle ache. The mechanism of action of capsaicin is not fully understood, current evidence suggests that capsaicin depletes substance P in peripheral sensory neurons, that induces the initial irritating sensation. This effect of capsaicin serves as the basis for its use as a valuable tool in investigating the roles of sensory neurons in biological functions (Buck *et al.* 1982).

Recently, clinical trials were conducted to alleviate cluster headaches (Fusco *et al.* 1994), desensitisation of nasal nerves to offset non-allergic rhinitis

(Riechelmann *et al.* 1994), lessen orofacial pain (Adekumle *et al.* 1994), prevent herpes flare-ups (Watson *et al.* 1988), reduce pain after burns and treat psoriasis (Glinski and Brodecka, 1994), using topical preparations containing capsaicin. Preparations of capsaicin currently available is shown in **Table 9.**

TABLE 9

BRAND/ PREPARATION	PERCENTAGE CAPSAICIN	COMPANY
AXSAIN(R)	0.075	Galen Pharma, IL
ZOSTRIX(R)	0.025	Gen Derm Corp., IL
CAPSAGEL TM	0.025-0.075	Iyata Pharmaceudicals, WI
BOSWELLIN (R)	0.025	Nature's Herb, Utah
PAIN DOCTOR TM	—	E. Fougera & Co.,NY
CAPZAISIN-P TM	0.025	Thompson Medical, FL
CAPZAISIN-HPTM	0.075	Thompson Medical, FL
MYOLAXIN	0.075	Geno Pharmaceuticals
MYOLAXIN-D	0.075	Geno Pharmaceuticals
NOVOLID GEL	0.25	Brown & Burn
AXANE CREAM	0.025	Medley
AXANE GEL	0.025	Medley

2.5 INNOVATIVE AND NOVEL USES OF CAPSAICIN

It has been suggested that supplementation of food with capsaicin, may help prevent aspiration pneumonia, the most common cause of death in the elderly (Sasaki *et al.* 1997). An added benefit of this diet is the improved clearance of the oesophagus (Gonzalez *et al.* 1998).

It was concluded in 1986 that those who eat plenty of hot, spicy food have a high metabolic rate and stay lean (Henry and Enreny, 1986). Cameron Smith and colleagues indicated the need to evaluate capsaicin as an antiobesity or slimming agent in humans. More recently, it has been shown that dietary hot peppers increase energy expenditure and diminish long term excess energy intake in humans (Lim *et al.* 1997) at the same time, also show enhanced oxygen uptake following capsaicin administration (Cameron - Smith *et al.* 1990).

It is noteworthy that capsaicin is bacteriocidal to *Helicobacter pylori* (Jones *et al.* 1997). In animal experiments, topical capsaicin protects against gastric ulcer formation (Abdel Salam *et al.* 1994). Thus, ingestion of chili peppers could have protective effect against gastroduodenal diseases. (Uchida M *et al.* 1991; Teny, CH *et al.* 1998).

3. MATERIALS AND METHODS

All experiments pertaining to the present study were carried out in the research laboratory of the Department of Biochemistry, University of Allahabad. The details of the materials used, experimental procedures followed and techniques adopted are described in this chapter.

1.3 MATERIALS USED

3.1.1 Chemicals

Chemicals and biochemicals were purchased from the following sources:

(i) Sigma Chemical Co., St. Louis, USA.

Name	Molecular weight	Product No.
Acetyl thiocholineiodide	289.2	A-5751
Adenosine triphosphate (disodium Salt)	551.1	A-2383
Albumin bovin serum (BSA) Fraction V	6600	A-9647
β-mercaptoethanol	78.13	M-6250
Capsaicin	305.4	M-2028
Capsazepine	376.9	C-901
Ouabain	728.8	O-3125

(ii) Hi-Media, Laboratories, Mumbai.

Name	Molecular weight	Product No.
Ammonium molybdate	1239.9	RM - 1018
ANSA (1-amino, 2 naphthol 4-sulphonic acid)	239.25	RM - 291
DTNB (5'5 dithiobis-2 nitro benzoic acid)	396.36	RM - 1677
EDTA (Ethylene diamine tetra acetic acid disodium salt)	292.5	RM - 1279
EGTA (Ethylene glycol-bis B-amino ethyl ether N,N,N',N'- tetraacetic acid)	380.4	RM - 1530
Heparin	—	RM - 639
Imidazole	68.08	RM - 559
Metaphosphoric acid sticks	98.0	RM - 1875
Potassium Chloride	74.56	RM - 698
Sodium Chloride	58.44	RM - 853
Sodium Lauryl sulphate	283.38	RM - 205
Tris-buffer	121.14	RM - 1218
Tris-HCl	157.6	RM - 613
Tertiary-Butyl hydroperoxide	90.12	RM - 2022

(iii) E. Merck India Ltd., Mumbai.

Name	Molecular weight	Product No.
Calcium Chloride	147.02	2380
Disodium hydrogen orthophosphate	178.0	17550
Potassium dihydrogen ortho phosphate	136.09	17546
Sodium Carbonate	105.99	17844
Sodium citrate	294.10	17556
Sodium dihydrogen orthophosphate	156.01	17845
Sodium hydroxide	40.00	17573
Sodium metabisulphite	190.00	27845
Sodium sulphite	126.04	17522
Tri-sodium citrate	294.10	17556

(iv) Coral Clinical Systems, Goa:

Name	Product No.
Ferricyanide-cyanide reagent (Drabkin's solution)	H-01000
Haemoglobin standard	H-01010

(v) Loba Cheme Indo Austranal Co. Mumbai :

Name	Product No.
Folin-Ciocalteu reagent	3870

All other chemicals were of AR grade or higher.

3.1.2 Selection of Subjects

Human volunteers of sound health in the age group of 25 - 40 years and having fasting glucose level 72.48 ± 12.36mg per 100 ml with no evidence of any clinical abnormality were used as control. None of the subjects had any family history of diabetes mellitus, hypertension and /or arthritis. The subjects were not suffering from pancreatitis and liver disorder. The BMI of the normal subjects was 22.1 ± 2.2 kg / m^2. Care was taken to select those subjects who were not on chemotherapy and were not receiving any hormonal treatment.

3.1.3 Washing of the Glasswares

All the glassware used during the experiments were first thoroughly washed with the detergent (Laboline) and then with chromic acid (potassium dichromate and conc. sulphuric acid) followed by prolonged drench in tap water and finally rinsed with double distilled water. These washed glasswares were then dried in hot oven.

3.1.4 pH measurement

Measurement of pH were performed on pH meter (Century - CP 901). The instrument was regularly calibrated with the help of standard buffer solutions (pH - 4.0, 7.0 and 9.2) prepared from standard buffer tablets.

3.1.5 Optical measurements

All routine colorimetric estimations were performed on spectrophotometer Spectrochem MK II, Enzyme assays were performed on ELICO-SL159, UV-VIS spectrophotometer.

3.1.6. *Blood Collection*

Venous blood was collected from normal healthy volunteers with informed consent, using acid-citrate dextrose (ACD) as an anti-coagulant (1.5:10 = ml anti-coagulant: ml blood).

The composition of ACD mixture is :

Citric acid ($C_6H_8O_7.H_2O$)	8.0 g
Sodium citrate ($C_6H_5O_7N_{a3}.2H_2O$)	22.0 g
Dextrose ($C_6H_{12}O_6.H_2O$)	24.5 g
Water (for make up)	1000 ml

For the determinaton of osmotic fragility blood was collected using heparin as an anti-coagulant (10 units/ml).

3.1.7 *Isolation of erythrocytes*

The blood sample obtained was centrifuged at 4°C (Remi C - 24 Cooling Centrifuge) for 10 minutes at 1000 g to remove plasma and buffy coat, care was taken to eliminate leucocyte contaminaton. The isolated erythrocytes were washed 4 to 5 times with 0.154 M NaCl (isotonic saline) in order to obtain completely washed pure erythrocytes free of leucocyte contamination.

3.1.8 *Isolation of red blood cell ghost*

The ghosts from leucocyte free red cells were obtained following the method of Marchesi and Palade (1967) that involves the principle of osmotic shock treatment with hypotonic and hypertonic buffers (pH 7.4). Washed erythrocytes were suspended in normal saline and frozen. Freezed erythrocytes were thawed and were hemolysed in hypotonic buffer (5 mM Tris-HCl buffer, 1 mM EDTA, pH 7.4). The hemolysed sample was kept for 30 min at 4° C with occasional stirring followed by centrifugation at 25 x 10^3 g for 30 minutes. After

centrifugation, the supernatant was aspirated out, the pellet (sediment) was again suspended in hypotonic buffer and the suspension was stirred for 5 min prior to centrifugation at the above mentioned speed for 10 min, the supernatant was discarded. The pellet (sediment) was now suspended in hypertonic buffer (50 mM Tris-HCl buffer, 1 mM EDTA and 500 mM NaCI, pH 7.4) and centrifuged with brake (mechanical shock) in the same manner as above. The pellet (sediment) was again suspended in hypotonic buffer, stirred for 10 min. and centrifuged. The treatment with hypotonic and hypertonic buffer was repeated until the ghost with no or minimum haemoglobin contamination was obtained.

3.2 METHODS

3.2.1 Determination of the ghost membrane protein content

The total protein content in the ghost samples was estimated by the method of Lowry *et al.* (1951) using Bovine Serum Albumin as the standard. Absorbance was measured at 620 nm.

Lowry reagent (alkaline copper sulphate) was prepared by mixing Solution A : Solution B : Solution C in the ratio of 1:1:98 freshly mixed just before estimation.

Solution A -2% w/v sodium potassium tartarate (Rochelle salt)

Solution B- 1% w/v copper sulphate

Solution C-2% w/v anhydrous sodium carbonate dissolved in 0.1 NaOH.

To 0.2 ml of the diluted ghost sample 0.8 ml of distilled water was added to make up the volume to 1 ml. To this 5 ml of freshly prepared Lowry reagent (alkaline copper sulphate) was added and thoroughly mixed by vortexing and allowed to stand for 10 minutes. After 10 minutes, 0.2 ml of the Folin-Ciocalteu reagent was added and vortexed, colour was allowed to develop and absorbance was measured after 30 min in spectrocolorimeter at 620 nm.

3.2.2 *Preparation of red cell hemolysate*

Red cell hemolysate was prepared as described by Beutler (1984). Packed red cells, obtained from 1 ml blood sample was suspended in approximately one volume of 0.154 M NaCl. To 0.2 ml of the suspension. 1.8 ml of β mercaptoethanol-EDTA stabilising solution (prepared by bringing 0.05 ml of β mercaptoethanol and 10 ml of neutralised 10% EDTA to a volume of 1 litre with water) was added. The tube containing the hemolysate was frozen and then thawed by placing the tube in a beaker containing water at room temperature. When the hemolysate was completely thawed, it was uniformly shaken and the tube was kept in ice water, where it was maintained at 0°C the hemolysate prepared in this way was referred to as 1:20 hemolysate.

3.2.3 *Estimation of haemoglobin*

Haemoglobin estimations were performed following the procedure of Beutler (1984). Ferricyanide-cyanide reagent (Drabkin's solution) prepared by dissolving 100 mg $NaCN^{-}$ and 300 mg $K_3Fe(CN)_6$ per litre water. For determination of haemoglobin, 0.2 ml of 1:20 hemolysate was added to 10 ml ferrcyanide-cyanide reagent. Fericyanide-cyanide reagent when added to diluted haemoglobin converts haemoglobin to cyanomethemoglobin and the optical density was measured at 540 nm within 5 minutes.

The spectrophotometer was calibrated by using commercially available cyanomethemoglobin standard. Such a standard was diluted in ferricyanide-cyanide solution to provide a range of concentrations from 0.005 - 0.045 g / 100 ml. When the optical density of the diluted standard solutions was plotted against

their haemoglobin concentration, a straight line was obtained. For ease of calculation, the absorbance value (OD_{540}) at which the calibration plot intersects the haemoglobin concentration of 10 mg / 100 ml was designated as A_1 and the haemoglobin calibration factor (F_{Hb}) was obtained as 1/100 A_1.

$$F_{Hb} = 1 / 100\ A_1$$

The concentration of haemoglobin in grams /100 ml in any sample is calculated by the formula:

$$\text{Haemoglobin (g / 100 ml)} = \frac{OD_{540} \times F_{Hb} \times (V_{Fe}+V_{Hb})}{V_{Hb}}$$

Where

OD_{540}	=	optical density at 540 nm
F_{Hb}	=	haemoglobin calibration factor
V_{Fe}	=	volume of ferricyanide - cyanide solution used
V_{Hb}	=	volume of haemoglobin solution used

3.2.4 Determination of acetylcholinesterase (AChE) activity

Red cell membrane acetylcholinesterase (AChE) activity was assayed in red cell hemolysates prepared following the method of Beutler. The procedure was the same as described by Beutler (1984) based on Ellman *et al.* (1961). The final asay mixture (5 ml) contained 0.1 M Tris-HCl, 0.5 mM EDTA (pH 8.0), 0.025 mM DTNB in 0.1% sodium citrate and 0.05 ml of 1:20 red cell hemolysate. After preincubation for 10 min at 37° C the reaction was initiated by

adding 0.25 ml of 10mM acetylthiocholine iodide. The increase in the optical density of the system was measured against that of the blank at 412 nm for 10 - 20 minutes.

The optical density of a blank assay, prepared by substituting the volume of hemolysate with 1:10 diluted β - mercaptoethanol-EDTA stabilising solution, was also read for 10 - 20 minutes at 412 nm and the blank rate was substracted from the rate measured in the presence of hemolysate.

The enzyme activity is calculated with the help of the formula:

$$E = \frac{10\ (\bullet OD_R - \bullet OD_B) \times V_{Hb} \times S}{F_{Hb} \times (V_{Fe} + V_{Hb}) \times OD_{540} \times \varepsilon \times V_H}$$

Where

$\bullet OD_R$ = optical density change of reaction system in 10 minutes

$\bullet OD_B$ = optical density change of blank system in 10 minutes.

V_{Hb} = volume of hemolysate added to ferricyanide-cyanide reagent

V_{Fe} = volume of ferricyanide-cyanide reagent used

F_{Hb} = the haemoglobin calibration factor

V_H = volume of hemolysate added to the reaction medium

OD_{540} = optical density of haemoglobin estimation

ε = millimolar extinction coefficient (13.6 for DTNB)

S = full scale reading of the instrument recorder

$$\text{Acetylthiocholine iodide} \xrightarrow{\text{AChE}} \text{thiocholineiodide} + \text{acetate ion}$$

$$\text{Thiocholine iodide} + \text{DTNB} \longrightarrow \text{5-thio-2-nitrobenzoic acid} + \text{oxidised thiocholineiodide (yellow)}$$

5-thio-2-nitrobenzoate ion imparts yellow colour to the assay medium having maximum absorption at 412 nm. A molar extinction coefficient of 1.36 x 10^{-4} litre mol^{-1} was used for the thionitrobenzoate ion at 412 nm.

Activity of acetylcholinesterase is expressed as I.U. i.e. µ mole of acetylthiocholineiodide hydrolysed per min per gram of haemoglobin at 37°C.

3.2.5 Assay of membrane bound (Na^+ / K^+ - ATPase)

Na^+ / K^+- ATPase activity was assayed by using the medium of Lukacovic *et al.* (1984) as described by Suhail and Rizvi (1987), with minor modifications, 1.0 ml of the assay mixture contained the following (final concentration): 140 mM NaCl, 20 mM KCl, 3mM $MgCl_2$, 30 mM imidazole (pH 7.25), 0.2 ml of the membrane solution containing 0.4 to 0.9 mg membrane protein per ml and ± 5 x 10^{-4} M ouabain. The above mixture was preincubated for 60 minutes at 37° C. After preincubation 6 mM ATP was added and the incubaton of the assay mixture was carried out at 37°C for 30 min.

Liberated phosphate was estimated by a modified method of Fiske and Subbarow (Fiske and Subbarow, 1925). The reaction was stopped by adding 0.7 ml of solution containing 0.5 M H_2SO_4, 0.5% ammonium molybdate and 2% SDS and mixing the solution thoroughly. After allowing it to stand for 10 minutes, 0.05 ml of colouring reagent containing 1.2% sodium metabisulphite, 1.2% sodium bisulphite and 0.2% ANSA was added and colour was allowed to develop for 25 minutes then centrifuged for 5 minutes after which absorbance was read at 650 nm.

Na^+ / K^+- ATPase activity is expressed as µ mol Pi liberated per milligram of membrane protein per hour at 37°C.

3.2.6 *Assay of membrane bound Ca^{++}- ATPase*

Ca^{++} - ATPase activity was assayed by the method of Raess and Vincenzi (1980) as described by Zaidi and Saleemuddin (1993). 1.0 ml of the assay mixture contained the following (final concentration): 80 mM NaCl, 15 mM KCl, 3 mM $MgCl_2$, 18 mM Tris-HCl (pH 7.4), 0.1 mM EGTA 0.1 mM Ouabain and 0.2 ml of the membrane solution containing 0.4 to 1.5 mg membrane protein per ml, ± 0.2 mM $CaCl_2$. The above mixture was preincubated for 60 minutes at 37° C, after pre incubation 3 mM ATP was added and the incubation of the assay mixture was carried out at 37°C for 30 minutes. The liberated Pi was estimated in both the assay control and experimental tubes and the Ca^{++} - ATPase activity was measured as the difference between the assay control and experimental tubes.

Liberated phosphate was estimated by modified method of Fiske and Subbarow (Fiske and Subbarow 1925). The reaction was stopped by adding 0.7 ml of solution containing 0.5 M H_2SO_4. 0.5% ammonium molybdate and 2% SDS and mixing the solution thoroughly. After allowing it to stand for 10 minutes, 0.05 ml of colouring reagent containing 1.2% sodium metabisulphite, 1.2% sodium sulphite and 2% ANSA was added and colour was allowed to develop for 25 minutes then centrifuged for 5 minutes and absorbance was read at 650 nm.

Ca^{++} - ATPase activity is expressed as µ mol Pi liberated per milligram of membrane protein per hour at 37°C.

3.2.7 *Estimation of erythrocyte (GSH) content*

Red blood lysate was prepared by mixing 0.2 ml of blood with 2.0 ml of

distilled water. Immediately 3 ml of the precipitating reagent (1.67 g glacial metaphosphoric acid, 0.2 ml of disodium EDTA and 30 g sodium citrate dissolved in 100 ml distilled water) was added to 2.0 ml of the lysate and allowed to stand for 5 minutes for the precipitation of proteins present in the red blood lysate. The above solution was filtered. To 2.0 ml of the filtrate 8.0 ml of 0.3 M Na_2HPO_4 solution was added and its absorbance was read against a blank containing (2:5) water diluted precipitating reagent (OD_1). The second optical density (OD_2) was immediately measured after addition of 1 ml of DTNB (containing 20 mg of DTNB per 100 ml of 1% sodium citrate solution) to both the blank and the above filtrate. A molar extinction coefficient of 13,600 was used when GSH reacts with DTNB.

Non-protein sulfdryl-groups of reduced glutathione (GSH) reduces 5, 5'-Dithiobis 2-nitrobenzoic acid (DTNB) forming a yellow coloured anionic product whose optical density is measured at 412 nm.

The concentration of glutathione (C) in micromoles per gram of haemoglobin (Hb) in the sample was calculated using the formula:

$$\frac{C}{1000} = \frac{(OD_2-OD_1)}{13600} \times \frac{E \times 11}{2} \quad \frac{\times 5}{2} \quad \frac{\times 100}{Hb}$$

Where

OD_2 = optical density read after addition of DTNB

OD_1 = optical density read before addition of DTNB

E = correction factor equals to 1 for a narrow slit in a 1 cm cuvette

Hb = haemoglobin concentration of sample in g / 100 ml

3.2.8 Determination of erythrocyte membrane sulfhydryl (SH) groups

The red blood cell membrane sulfhydryl group was determined following the method of Kitajima *et al.* 0.2 ml of ghost membrane was mixed with 0.3 ml of 20% SDS and 2.8 ml of 0.1M sodium phosphate buffer containing 0.1 mM EDTA (pH 8.0). The above mixture was incubated at 37°C for 60 minutes. The solution was divided into two parts (3.0 ml and 0.3 ml). To the 3.0 ml of the solution, 0.1 ml of the DTNB was added and again incubated for 15 minutes at 37°C and its absorbance was read against reference sample, which did not contain ghost membrane.

To the 0.3 ml of the solution, protein content was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard. Absorbance was measured at 620 nm.

Sulfhydryl groups of the membrane reduce 5,5'-Dithiobis 2-nitro benzoic acid (DTNB) forming a yellow coloured anionic product whose optical density is measured at 412 nm. A molar extinction co-efficient of 13,600 was used when SH group reacts with DTNB.

The concentration of sulfhydryl group in nanomoles per milligram of protein in the sample was calculated using the formula :

$$\text{SH group} : \quad \frac{C_{SH}\ (\bullet A)}{C\ \text{protein}\ (\bullet B)}$$

Where

C_{SH} (•A) : Optical density of SH concentration read at 412 nm.

C protein (•B) : Optical density of protein concentration read at 620nm.

3.2.9 Determination of red cell osmotic fragility

Heparinized blood was added to hypotonic solutions of varying concentrations of NaCl in the proportion of 1 - 100 and the extent of hemolysis, after 30 min was measured colorimetrically at 540 nm. The procedure adopted was the same as described by Dacie and Lewis (1984).

A stock solution of buffered NaCl was prepared by dissolving 5g NaCl, 1.3655 g Na_2HPO_4 and 0.243 g NaH_2PO_4. $2H_2O$ in water and raised upto 100 ml. The solution was osmotically equivalent to 10% NaCl. For preparing hypotonic solutions, firstly 1% solution was made from the stock 10% solution by dilution with double distilled water. Further dilutions were made to prepare solutions osmotically equivalent in the range of 0.85% to 0.1%.

To 5 ml of hypotonic solutions, 0.05 ml of heparinized blood was added and mixed well immediately. The tubes were allowed to stand at room temperature for 25 min and then centrifuged for 5 min at 100 g. The amount of hemolysis in each tube was compared with that in 100% lysis tube (0.1% NaCl) colorimetrically at 540 nm. The supernatant from 0.85% NaCl tube was used as the blank.

Extent of hemolysis was calculated by dividing the absorbance of varying hypotonic solution by absorbance of solution showing maximum hemolysis (0.1% NaCl).

$$\text{Extent of hemolysis} = \frac{\text{OD of supernatant of varying NaCl\%}}{\text{OD of supernatant with maximum lysis (0.1\% NaCl)}} \times 100$$

The erythrocyte hemolysis curve was prepared by plotting percent of NaCl solution on X-axis and per cent hemolysis on Y-axis.

3.2.10 *Experiments with capsaicin and capsazepine*

In vitro experiments were carried out by adding appropriate concentration of capsaicin and / or capsazepine, dissolved / diluted in ethanol/distilled water, to the whole blood and incubating at 37° C for 15, 30, 60, 90 and 120 minutes prior to the isolation of erythrocytes.

In experiments involving isolated red blood cell membrane the following incubation procedure was adopted. *In vitro* experiments were carried out by adding capsaicin and / or capsazepine, dissolved / diluted with ethanol / distilled water to get the appropriate concentration (10^{-5} - 10^{-8} M), to the enzyme assay medium incubated at 37°C for 60 minutes. The incubation time was varied between 0 -120 minutes during time dependent study prior to the enzyme assay.

Parallel control experiments were also performed where capsaicin and / or capsazepine were replaced with equal amount of solvent in which capsaicin and capsazepine solution was made.

3.3 *STATISTIC METHODS USED IN THE ANALYSIS OF DATA*

3.3.1 *Measure of central tendency*

An average is a typical value, which tends to sum up or describes the mass of the data. The average is the measure of the location of central tendency. Several types of averages can be defined but the most commonly used are the arithmetic mean, the median and the mode. In our experiments we have used arithmetic mean as a measure of central tendency for interpretation of data.

3.3.1.1 Arithmetic mean

The arithmetic mean is the calculated average. The arithmetic mean of a set of numbers x_1, x_2, x_3 ..... x_n, is denoted by x (x-bar) and is calculated as :

$$x = \frac{x_1 + x_2 + x_3 \ldots\ldots\ldots\ldots\ldots + x_n}{n}$$

$$= \frac{1}{n} \sum_{i=1}^{n} X_i$$

$$\text{or } X = \frac{\sum x}{n}$$

3.3.2 Measure of dispersion of variation

There are various measures of dispersion used such as range, standard deviation, standard error, coefficient of variation etc., we have used standard deviation as a measure of dispersion for interpretation of data.

3.3.2.1 Standard deviation

The degree to which numerical data tends to spread about an average value is called the variation or dispersion of data, standard deviation is most commonly used as a measure of dispersion of variation.

The standard deviation of a set of 'n' number x_1, x_2, x_3..... x_n, with mean value x is donated by SD and is calculated by

$$S.D. = \sqrt{(x_1 - x)^2 + (x_2 - x)^2 + (x_3 - x)^2 + \ldots\ldots\ldots\ldots (x_a - x)^2, n-1))}$$

3.3.3 Test of significance ('t' test)

There are various types of problems for which tests of significance are used for drawing conclusion for two sample means. The most commonly used test in assessing the significance of difference between two sample mean is the 't' test.

For two samples with mean x_1, x_2 and number of sample n_1, and n_2, respectively the 't' value is calculated as :

$$'t' = \frac{(x_1 - x_2)}{\sqrt{\left(\frac{1}{n_1} + \frac{1}{n_2}\right) SD^2}}$$

$$'t' = \frac{(x_1 - x_2)}{\text{Standard error (SE)}}$$

$$SD^2 = \frac{1}{(n_1+n_2-2)} \left[(n_1-1)\, SD_1^2 + (n_2-1)\, SD_2^2\right]$$

where $SE = \sqrt{\left(\frac{1}{n_1} + \frac{1}{n_2}\right)\left(\frac{(n_1-1)SD_1^2 + (n_2-1)SD_2^2}{n_1+n_2-2}\right)}$

Degree of freedom for the above 't' test

$= n_1 + n_2 - 2$

From the calculated 't' value p is calculated corresponding to its degree of freedom from the **Table 10**. We have used p value less than 0.01 as a measure of significance.

TABLE - 10

Distribution of 't' probability :

Degree of Freedom	Calculated 't' values		
p	0.05	0.01	0.001
1	12.706	63.657	636.619
2	4.303	9.925	31.698
3	3.182	5.841	12.924
4	2.776	4.604	8.610
5	2.571	4.032	6.869
6	2.447	3.707	5.959
7	2.365	3.449	5.408
8	2.306	3.335	5.041
9	2.262	3.250	4.780
10	2.228	3.169	4.587
11	2.201	3.106	4.437
12	2.179	3.055	4.318
13	2.160	3.012	4.221
14	2.145	2.977	4.140
15	2.131	2.947	4.073
16	2.120	2.921	4.015
17	2.110	2.898	3.965
18	2.101	2.878	3.922
19	2.039	2.861	3.983
20	2.086	2.845	3.850
22	2.074	2.819	3.792
24	2.064	2.797	3.745
26	2.056	2.779	3.707
28	2.048	2.763	3.674
30	2.021	2.750	3.646
60	2.000	2.660	3.460
120	1.980	2.617	3.737
Infinite	1.960	2.576	3.291

3.3.4 Analysis of variance

When we wish to compare the mean of two samples 't' test is most suitable but when it is required to test the differences between more than two samples, probably the most appropriate technique is called analysis of variance (ANOVA).

The need for applying ANOVA is to estimate whether the variation in observed values is due to sample differences or due to random variability within the samples. By comparing these two variations we can determine the importance of the sample differences. From ratio of these two variations we can determine the importance of the sample differences. The ratio of these two types of variations is called 'F'. A high value of this F-value indicates the significant differences between the samples. The observed value of F-statistics can be compared to the critical value of F-at 5% level from the F distribution table.

3.3.4.1 Performing the test of ANOVA

In performing the test of analysis of variance there are few important statistical calculations.

3.3.4.2 Sum of square

The value of the sum of squares (S.S.) can be computed as the sum of the squares of deviation of the values from the mean of the sample.

$$S.S. = \bullet (X-X)^2 \text{ or,}$$

$$S.S. = \bullet_X 2 - \frac{(\bullet X)^2}{n}$$

3.3.4.3 Mean square

This is the mean of all the sum of squares and obtained by dividing the S.S. by its appropriate degrees of freedom. This quantity is equal to the variance as explained earlier.

$$\text{Mean square (M.S.)} = \frac{\text{S.S.}}{\text{d.f.}}$$

Three quantities are first obtained in the ANOVA.

(i) Total sum of squares.

(ii) Between sample sum squares.

(iii) Residual sum of squares.

In the total S.S., x and n can be calculated as :

$$\bullet x = T_1 + T_2 + T_3 \ldots T_n$$

$$n = n_1 + n_2 + n_3 \ldots n_n$$

Where

n = no. of sample.

T = Total of sample.

x = mean of sample.

Total S.S. calculated by S.S. $= \bullet x^2 - \frac{(x)^2}{n}$

$$= S - \frac{T^2}{n}$$

In between sample S.S.

$$= \bullet \frac{T_i^2}{n_i} - \frac{T^2}{n}$$

Degree of freedom

= Total no. of samples sets - 1

In the residual S.S. (within sample S.S.)

The quantity denotes the variance within the sample can be calculated as :

Residual S.S. = Total S.S. - Between samples S.S.

Degree of freedom = Total no. of observations - Total No. of Sample sets

To show complete ANOVA test, we use a standard form of a table which has normally five columns and the **Table 11** is known as the analysis of variance table.

3.3.4.4 Analysis of variance table

TABLE - 11

Source of variance	Degree of freedom d.f.	Sum of squares (S.S.)	Mean Square $M.S.=\frac{S.S.}{d.f.}$	Variance ratio F
Between sample	x_1	y_1	x_1/y_1	a
Residuals	x_2	y_2	x_2/y_2	b
Total	$x_1 + x_2$	$y_1 + y_2$	a/b	F

The statistics 'F' is calculated from

$$F = \frac{\text{Between sample variance (M.S.)}^a}{\text{Within sample variance (residual M.S.)}^b}$$

From the **Table 12** we find the degree of freedom respectively for the numerator and the denominator. The value indicates that the different conditions significantly differs from each other or not.

TABLE - 12

Values of F (Variance Ratio)

n_2	n_1 1	2	3	4	5	6	8	12	24	α
1	161 40	199 50	215.70	224.60	230.20	234.00	238.90	243.90	249.00	254.30
2	18.51	19.00	19.16	19.25	19.30	19.33	19.37	19.41	19.45	19.50
3	10.13	9.55	9.28	9.12	9.01	8.94	8.84	8.74	8.64	8.53
4	7 71	6 94	6.59	6.39	6.26	6.16	6.04	5.91	5.77	5.63
5	6.61	5.79	5.41	5.19	5.05	4.95	4.82	4.68	4.53	4.36
6	5.99	5.14	4.76	4.53	4.39	4.28	4.15	4.00	3.84	3.67
7	5.59	4.74	4.35	4.12	3 97	3.87	3.73	3.57	3.41	3.23
8	5.32	4.46	4.07	3.84	3.69	3.58	3.44	3.28	3.12	2.93
9	5.12	4.26	3.86	3.63	3.48	3.37	3.23	3.07	2.90	2.71
10	4.96	4.10	3.71	3.48	3.33	3.22	3.07	2.91	2.74	2.54
11	4.84	3.98	3.59	3.36	3.20	3.09	2.95	2.79	2.61	2.40
12	4.75	3.88	3.49	3.26	3.11	3.00	2.85	2.69	2.50	2.30
13	4.67	3.80	3 41	3.18	3 02	2.92	2.77	2.60	2.41	2.21
14	4.60	3.74	3.34	3.11	2 96	2.85	2.70	2.53	2.35	2.13
15	4.54	3.68	3.29	3 06	2.90	2.79	2.64	2.48	2.29	2.07
16	4.49	3.63	3.24	3.01	2.85	2.74	2.59	2.42	2.24	2.01
17	4.45	3.59	3.20	2.96	2.81	2.70	2.55	2.38	2.19	1.96
18	4.41	3.55	3.16	2.93	2.77	2.66	2.51	2 34	2.15	1.92
19	4.38	3 52	3.13	2.90	2.74	2.63	2.48	2.31	2.11	1.88
20	4.35	3.49	3.10	2.87	2.71	2.60	2.45	2.28	2.08	1.84
21	4.32	3.47	3.07	2.84	2.68	2.57	2.42	2.25	2.05	1.81
22	4.30	3.44	3.05	2.82	2.66	2.55	2.40	2.23	2.03	1.78
23	4.28	3.42	3.03	2.80	2.64	2.53	2.38	2.20	2.00	1.76
24	4.26	3.40	3.01	2.78	2.62	2.51	2.36	2.18	1.98	1.71
25	4.24	3.38	2.99	2.76	2.60	2.49	2.34	2.16	1.96	1.71
26	4.22	3.37	2 98	2.74	2.59	2.47	2.32	2.15	1.95	1.69
27	4.21	3.35	2.96	2.73	2.57	2.46	2.30	2.15	1 93	1.67
28	4.20	3.34	2.95	2.71	2.56	2.44	2.29	2.12	1.91	1.65
29	4.18	3.33	2.93	2.70	2.54	2.43	2.28	2.10	1.90	1.64
30	4.17	3.32	2.92	2.69	2.53	2.42	2.27	2.09	1.89	1.62
40	4.03	3.23	2.84	2.61	2.45	2.34	2.18	2.00	1.79	1.51
60	4.00	3.16	2.76	2.52	2.37	2.25	2.10	1.92	1.70	1.39
120	3.92	3.07	2.68	2.45	2.29	2.17	2.02	1.83	1.61	1.25
α	3.84	2.99	2.60	2.37	2.21	2.09	1.94	1.75	1.52	1.00

4. *AIM OF THE STUDY*

The present study aims to investigate in depth the *in vitro* effects of capsaicin and / or capsazepine on human erythrocyte and its mechanism of action in an effort to understand the interaction of capsaicin with plasma membrane of non-neuronal cells in the human body. During the study, experiments were carried out on the *in vitro* effect of capsaicin and / or capsazepine on erythrocyte membrane bound enzymes and membrane transports processes, membrane surface -SH groups, intracellular reduced glutathione and osmotic fragility of erythrocytes.

The objectives of the present study, in brief, is to investigate the following:

1. Effect of capsaicin and / or capsazepine on erythrocyte membrane transport processes :

   (a) Na^+ / K^+ - ATPase

   (b) Ca^{++} - ATPase

2. Effect of capsaicin and / or capsazepine on membrane bound enzymes which are known to be modulated by changes in membrane fluidity:

   - Acetylcholinesterase.

3. Effect of capsaicin and / or capsazepine on erythrocyte membrane bound -SH group.

4. Effect of capsaicin and / or capsazepine on intracellular reduced glutathione (GSH) content in erythrocytes.

5. Dose - responsive effect of capsaicin and / or capsazepine on studies detailed in objectives 1,2,3 and 4.

6. Time - dependence effect of capsaicin and / or capsazepine on studies detailed in objectives 1,2,3 and 4.

The erythrocyte was chosen in the present study for various reasons:

(i) The erythtocyte being a convenient and easily available model, devoid of cellular organelles, is highly suitable for cell surface biochemical studies.

(ii) It acts as a suitable biosystem to investigate the probable biochemical alterations at the cell membrane level.

(iii) The different analytical methods, which are commonly employed in biomembranology especially the erythrocyte membrane are universally standardized, accepted and reproducible.

The human erythrocyte membrane is probably the most extensively studied biological membrane. This is not only because erythrocyte membranes can be prepared in large quantities in homogenous form by simple hypotonic lysis procedures, (Dodge *et al.* 1963), but also because they represent a readily accessible cell for studies on the molecular basis of human disease states (Sauberman *et al.* 1979).

This study has focussed on the effect of capsaicin and / or capsazepine on human erythrocytes in an effort to understand the non-neuronal effects. The study may be important since capsaicin is being increasingly used as an analgesic in form of topical creams/ointments. The present study was also expected to provide an understanding of the physiological impact of high capsicum (capsaicin) consumption by humans in tropical regions of the world.

5. RESULT & DISCUSSION

5.1 ACETYLCHOLINESTERASE

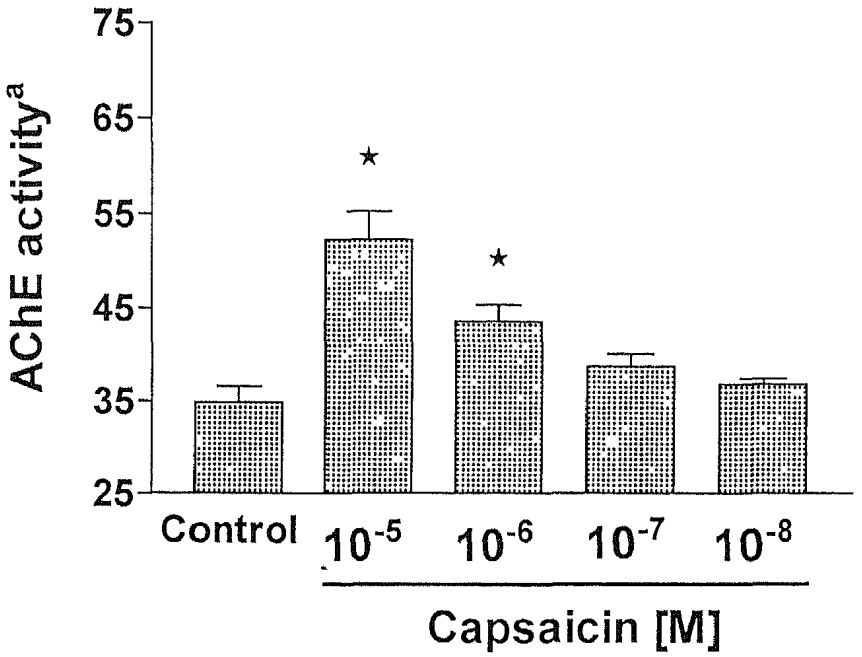
Acetylcholinesterase (Acetylcholine hydrolase E.C. 3.1.1.7) is a key enzyme in cholinergic neuro transmission. It is localised in certain areas of the central nervous system, organs that are controlled by the autonomic (parasympathetic) nervous system as well as in the erythrocytes of mammals (Massoulie and Bonn, 1982). In brain, it is present at neuro-muscular junctions where impulses are transmitted from nerves to skeletal muscle fibres. At these junctions, a neuro transmitter substance acetylcholine is present and the enzyme acetylcholinesterase (AChE) which has remarkably high specificity for this substance causes specific and rapid hydrolysis of acetylcholine resulting in the inactivation of this neurotransmitter. This efficient inactivation prevents re-excitation after the stimulated cell has recovered from the first action potential. The importance of the enzyme is best documented by the fact that irreversible inhibition induces a constant exitation of the parasympathetic nervous system and of muscle tisues which ultimately leads to death.

AChE from erythrocyte membranes has been purified and reasonably well characterized (Ott *et al.* 1975). The activity of human erythrocyte membrane AChE of normal (control), capsaicin and/or capsazepine treated is given in **Table 13.**

In vitro incubation with capsaicin resulted in significant activation ($p < 0.01$) of human erythrocyte membrane AChE activity.

The concentration - dependent effect of capsaicin (10^{-5} - 10^{-8} M) is shown in **Figure 10**. Maximum increase in the activity of AChE is observed at 10^{-5} M

Figure 10



Concentration-dependent effect of capsaicin on erythrocyte membrane acetylcholinesterase (AChE) activity

[a] Activity of erythrocyte membrane AChE is expressed as I.U.
(μM of acetylthiocholineiodide hydrolysed per min per gram of hemoglobin at 37° C).
* p (< 0.01) as compared to control.
Values are mean ± SD of 4 -5 independent experiments.

capsaicin whereas less activation is observed at succeeding lower concentration with almost no effect at 10^{-8} M capsaicin.

The time-dependent effect of capsaicin (10^{-6} M) on the activity of human erythrocyte membrane AChE is shown in **Figure 11.**

Capsaicin's effect increases with time reaching a maximum after 60 min of incubation. Further increase in incubation time resulted in diminished effect and no activation of enzyme activity was evident after 120 min.

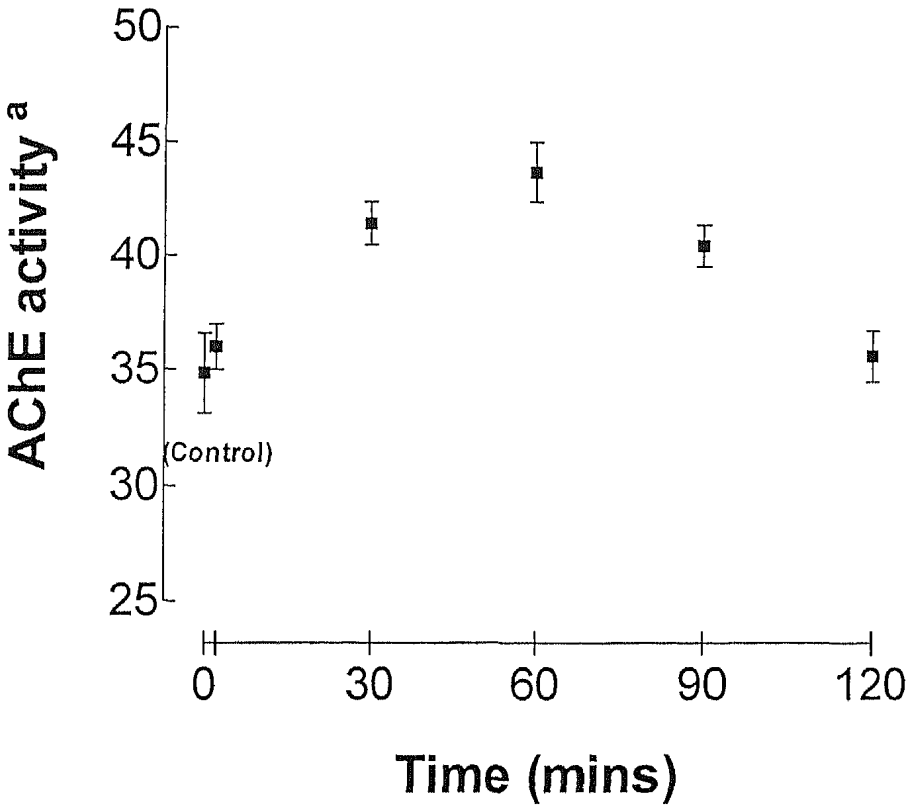
In vitro capsazepine (10^{-5} M) treatment did not affect AChE activity. However, *in vitro* incubation of erythrocyte with capsaicin (10^{-5} M) along with capsazepine (10^{-5} M) caused a significant activation of AChE activity as given in **Table 13.**

No significant change in the activity of AChE after capsazepine (10^{-5} M) treatment clearly signify that capsazepine did not have any significant effect on human erythrocyte AChE activity.

Capsaicin is a lipophilic molecule and is reported to interact with the plasma membrane (Buck and Burks, 1986), interaction with the plasma membrane causes increase in membrane fluidity, an effect which is concentration dependent (Meddings *et al.* 1991).

The increase in the AChE may be explained on the basis of capsaicin - induced alteration in membrane fluidity. The activity of red cell AChE is known to co-relate with membrane fluidity (Kamada *et al.* 1992).

Figure 11



Time-dependent effect of capsaicin on erythrocyte membrane acetylcholinesterase (AChE) activity

a Activity of erythrocyte membrane AChE is expressed as I.U.
(μM of acetylthiocholineiodide hydrolysed per min per gram of hemoglobin at 37° C).
Values are mean ± SD of 4 -5 independent experiments.

TABLE - 13

***In vitro* effect of capsaicin and / or capsazepine on erythrocyte membrane acetyl cholinesterase (AChE) activity.**

Conditions	n	AChE activity[a]
Control	11	34.96 ± 3.10
+ Capsaicin (10^{-5} M)	13	52.34 ± 3.72 *
+ Capsazepine (10^{-5} M)	10	35.61 ± 3.42
+ Capsaicin (10^{-5} M) and Capsazepine (10^{-5} M)	12	45.74 ± 2.79*

n number of subjects.

a Activity of AChE is expressed as µM of acetyl thiocholine iodide hydrolysed / min / g of Hb at 37°C.

* p (<0.01) as compared to control
Values are mean ± S.D. of 4 - 5 independent experiment in each group.

The importance of the erythrocyte membrane AChE lies in the fact that many of its properties have been found similar to the purified enzyme obtained from the brain tissue (Sorenson *et al.* 1982). For this reason, it appears that erythrocyte membrane AChE can substitute for this nerve tissue enzyme as a valid model system in *in vitro* studies, despite the fact that uptill now the biological function and significance of the erythrocyte AChE is still unknown (Ott, 1985). It has been recently suggested that AChE can be an excellent marker for RBC aging in man (Prall *et al.* 1998).

AChE activity of erythrocytes is reported to undergo circadian oscillations over a 24 hour period (Morariu, 1996). A signficantly elevated RBC AChE activity has been recorded in pregnant women (Rumenjak, 1998).

Our observation of capsaicin - induced transient (time dependent) activation of erythrocyte AChE activity is an interesting finding and may have important implications.

Although, it is difficult on the basis of our present observations to explain how capsaicin - induced elevated AChE activity might affects humans, these finding may help to explain certain neuronal effects of capsaicin. In view of increasing use of vanilloids as therapeutic agents, the present findings suggest that vanilloids may also cause side-effects.

5.2 CALCIUM ADENOSINE TRIPHOSPHATASE

Calcium adenosine triphosphatase (Ca^{++} - ATPase) or the calcium pump is an integral membrane protein found in all plasma membranes. The calcium pump transport Ca^{++} out of the cell involving high phosphate bound energy of ATP which is essential for maintaining Ca^{++} concentration of the cell cytoplasm. Membrane bound calcium transporting proteins are important in regulating various signal functions of calcium and regulation of this calcium is performed by Ca^{++} - ATPase or the calcium pump (Carafoli, 1992).

The pump was discovered in erythrocytes but is an obligatory component of eukaryotic plasma membranes. Its properties are essentially similar in all plasma membranes but subtle differences may exist (Kessler *et al.* 1990).

Ca^{++} - ATPase molecule is so oriented that its active component is on the inner side of the membrane. It is, in turn, the immediate phospholipid milieu

surrounding the Ca^{++} - ATPase molecule that exerts the greatest influence on the enzyme activity (Warren *et al.* 1975; Levy *et al.* 1990).

The erythrocyte membrane associated calcium pump maintains the low intracellular Ca^{++} concentration with remarkable efficiency at the expense of cellular ATP (Schatzmann, 1975). When the pump fails as in sickle cells or in some hereditary metabolic diseases, the intracellular Ca^{++} level rises significantly and triggers a number of processes (Zaidi and Saleemuddin, 1991). Almost all senescent related alterations including alteration in shape (Palek *et al.* 1974; Sheetz and Sawyer, 1978), disturbances in phospholipid organization (Allan, 1976), increase in cell rigidity (Caraway *et al.* 1971), degradation of membrane glycopeptides (Croall, 1986), peroxidation of lipids (Jain and Shohet, 1981) are either directly or indirectly brought about by high Ca^{++} levels. In view of its ability to deplete celular ATP and induce oxidative stress, elevated Ca^{++} level may cause aggregation of band 3 (Lutz, 1981, Low *et al.* 1985).

The human red cell Ca^{++} - ATPase is stimulated by direct interaction with Calmodulin (CaM) (Gopinath and Vincenzi, 1977). This has been exploited to purify the pump, first from erythrocytes (Niggli, 1979) and then from other plasma membranes using CaM columns. CaM increases both Ca^{++} affinity and maximal rate of hydrolysis (Stieger and Luterbacher, 1981). Such an effect is thought to occur through Ca^{++}- mediated CaM binding to an enzyme domain presumably affecting two different portions of the ATPase (Enyedi, 1989). This domain is located on a cytoplasmic segment which bears the putative Ca^{++} binding sites (James, 1988). The activity of human erythrocyte membrane Ca^{++} - ATPase of normal (control), capsaicin and /or capsazepine treated is given in

Table 14.

In vitro incubation with capsaicin resulted in significant activation ($p<0.01$) of human erythrocyte membrane Ca^{++} - ATPase activity.

The concentration - dependent effect of capsaicin is shown in **Figure 12.** Maximum increase in the activity of Ca^{++} - ATPase is observed at 10^{-5} M capsaicin, which decreased at lower concentration with almost no effect at 10^{-8} M capsaicin.

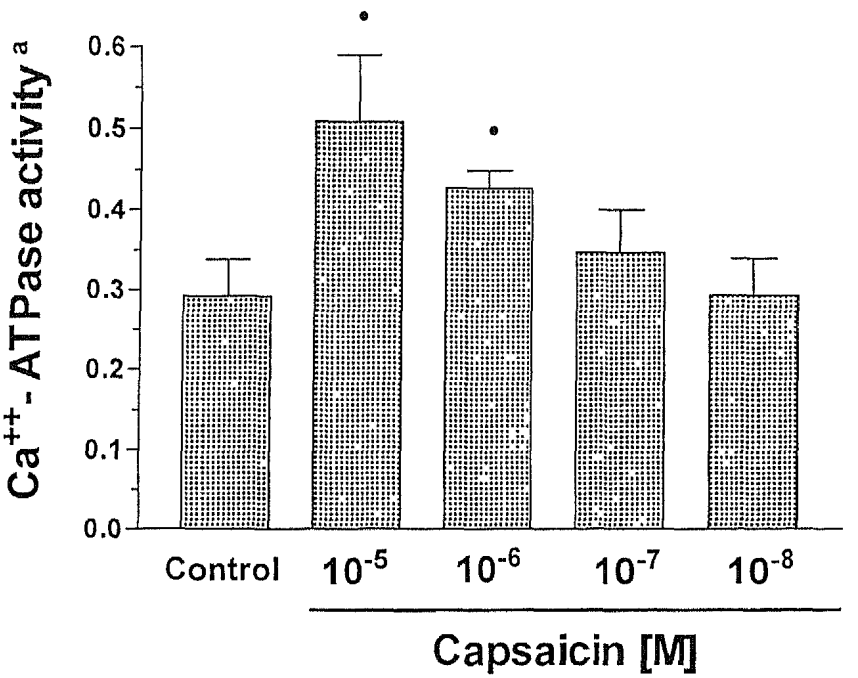
The time-dependent effect of capsaicin (10^{-6} M) on the activity of human erythrocyte membrane Ca^{++} - ATPase is shown in **Figure 13.** The effect of capsaicin was fast, activation of enzyme activity was observed within minutes of incubation, reaching a maximum after 60 min of incubation. Further increase in incubation time resulted in diminished effect and enzyme activity returned to normal level after 120 min of incubation.

In vitro capsazepine (10^{-5} M) treatment did not affect Ca^{++} - ATPase activity. However, *in vitro* incubation of erythrocyte with capsaicin (10^{-5} M) along with capsazepine (10^{-5} M) caused activation of enzyme Ca^{++} - ATPase activity as given in **Table 14.**

No significant change after capsazepine (10^{-5} M) treatment clearly signify that capsazepine did not have any significant effect on human erythrocyte Ca^{++}-ATPase activity.

The Ca^{++} - ATPase transports calcium out of the cell in a process coupled with the hydrolysis of ATP and in the process maintains cell calcium homeostasis (Carafoli, 1992). Capsaicin is a lipophilic molecule, which activates ion channels

Figure 12



Concentration-dependent effect of capsaicin on erythrocyte membrane calcium adenosine triphosphatase (Ca^{++}-ATPase) activity

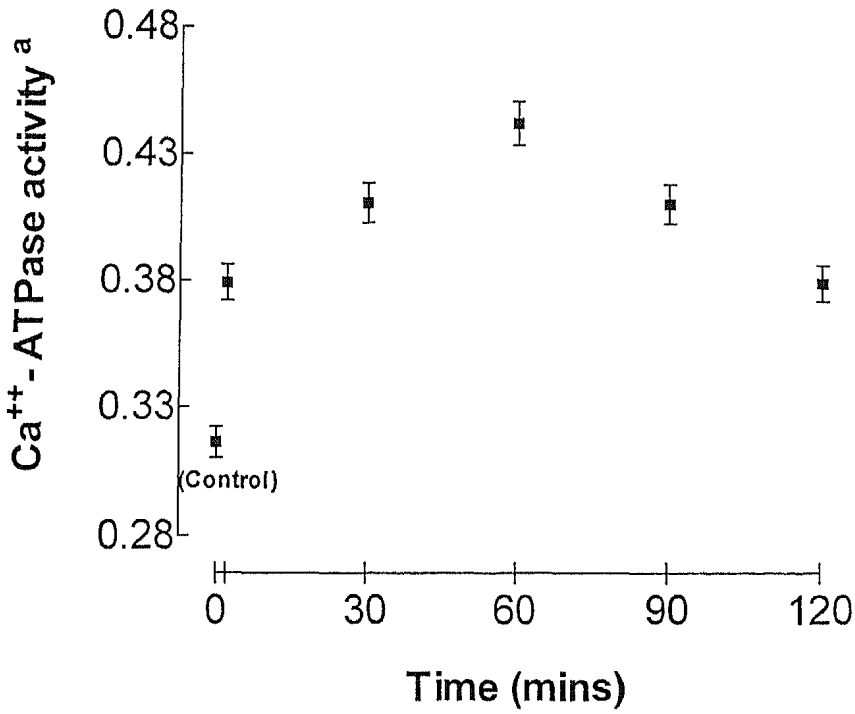
a Activity of erythrocyte membrane Ca^{++}-ATPase is expressed as μM of Pi released per hour per milligram of protein at 37° C.

* p (< 0.01) as compared to control.

Values are mean ± SD of 4 -5 independent experiments.

by directly binding from either exterior or interior of the cell. Activation of the ion-channel make Ca^{++} to flow from sera or plasma (where they are found at high concentration; 2 mM) into the intracellular space (where the concentration is 100 nM) (Dray *et al.* 1980). Opening of the ion channel thus not only initiates nerve impulses by depolarizing the membrane potential, but it also raises the level of intracellular calcium considerably (David, E Clapham, 1997). Recent researches have shown that capsaicin interacts with the plasma membrane and alters its fluidity (Meddings, 1991). Capsaicin is reported to incorporate in the plasma membrane, perturbing the packing of lipids and affecting their thermotropic properties (Aranda, 1995), this may explain the effect of capsaicin on Ca^{++} - ATPase activity of human erythrocytes.

Figure 13



Time-dependent effect of capsaicin on erythrocyte membrane calcium adenosine triphosphatase (Ca^{++}-ATPase) activity

a Activity of erythrocyte membrane Ca^{++}-ATPase is expressed as μM of Pi released per hour per milligram of protein at 37° C.
Values are mean ± SD of 4 -5 independent experiments.

TABLE - 14

***In vitro* effect of capsaicin and / or capsazepine on erythrocyte membrane calcium adenosine triphosphatase (Ca^{++}- ATPase) activity.**

Conditions	n	Ca^{++} - ATPase activity[a]
Control	09	0.3120 ± 0.009
+ Capsaicin (10^{-5} M)	11	0.5460 ± 0.016*
+ Capsazepine (10^{-5} M)	08	0.3051 ± 0.01
+ Capsaicin (10^{-5} M) and Capsazepine (10^{-5} M)	12	0.4972 ± 0.013*

n number of subjects.

a Activity of Ca^{++}-ATPase is expressed as µM of Pi liberated/ hour/mg of protein at 37°C.

* p (<0.01) as compared to control

Values are mean ± S.D. of 4 - 5 independent experiment in each group.

It is however, not clear whether the activation of Ca^{++} - ATPase in red cell is a compensatory response of the cell to increased calcium influx due to opening of the ion channel by capsaicin or a result of a direct effect of capsaicin on red cell membrane.

The importance of transient change in the intracellular Ca^{++} level in presence of capsaicin may have some important physiological implications. Calcium acts as a second messenger in the mechanism of action of several hormones and it regulates the activity of several enzymes.

The observation of significant change in the activity of the erythrocyte membrane Ca^{++} - ATPase after *in vitro* capsaicin treatment shows that this compound has significant effect on Ca^{++} - ATPase activity of erythrocyte membrane.

5.3 Sodium Potassium Adenosine Triphosphatase

The sodium potassium adenosine triphosphatase (Na^+ / K^+ - ATPase - E.C. 3.6.1.37) also known as sodium pump, acts as an enzymatic transport system and represents a universal mechanism of animal cell membrane that transport 3 Na^+ outwards from the cell and $2K^+$ inwards the cell across the cell membrane irrespective of the ionic gradient. The energy for the transfer of Na^+ and K^+ across the cell membrane is derived from the hydrolysis of ATP, which splits off into ADP and inorganic phosphate (Pi), the reaction is catalyzed by intracellular Mg^{++}. One mole of ATP is broken down for the transport of 3 Na^+ outwards from the cell and $2K^+$ inwards the cell.

Na^+ / K^+ - ATPase is specifically inhibited by binding of extracellular cardioactive steroids such as ouabain, a glycoside and digitalis, a cardiotonic drug, which inhibits the Na^+ / K^+- pump by blocking the step of dephosphorylation.

Na^+/K^+ - ATPase contains 2 polypeptides, the α and β sub units. The α sub units is the catalytic sub unit. It binds ATP or ouabain and accepts a covalent phopshate group from ATP or inorganic phosphate (Pi) as an intermediate in the reaction. Since the same molecule binds intracellular ATP or extracellular ouabain, the molecule spans the membrane. The β sub unit is a glycoprotein showing microheterogeneity . It has been suggested that it serves as a stable fulcrum against which the catalytic subunit executes its conformational changes more precisely than it would were it acting alone (Post, 1983).

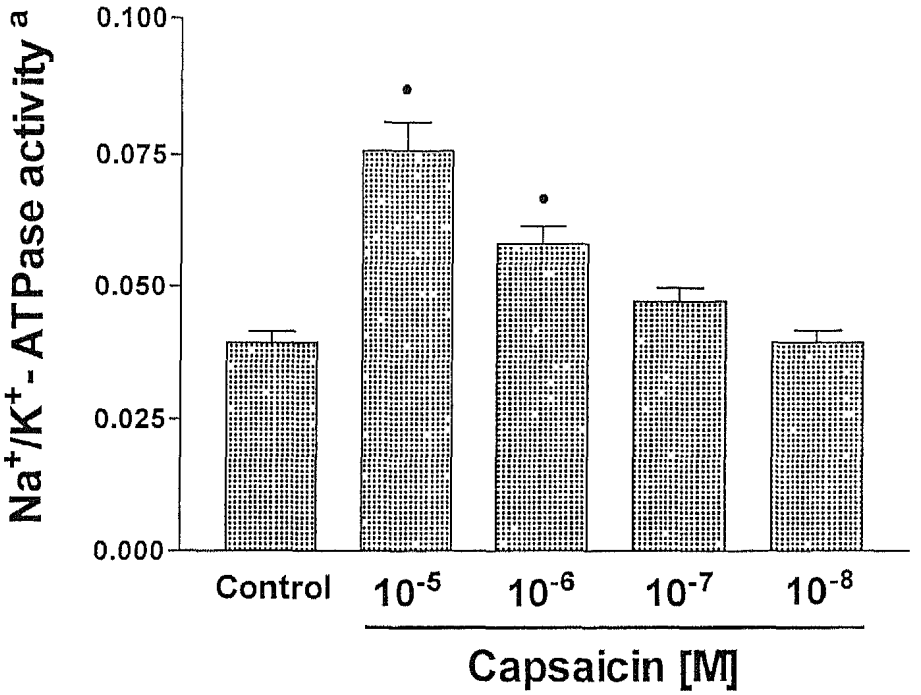
A number of cell functions depend on the movement of Na^+ and K^+ across the cell membrane. Entry of sodium and exit of potassium from the cell across cell membranes takes through ion-selective channels causing the generation of action potentials. At the same time many transport processes such as co-transport of nutrients, amino acids, sugars, neurotransmitters and selective anions (Schwartz *et al.* 1972) and counter transport of hydrogen ions and calcium ions (Blaustein, 1974) takes place due to inwardly directed electrochemical gradient for sodium. **(Figure 9)**. A number of specialized cellular functions such as excitability, regulation of cell volume, osmotic pressure, muscle contraction and cytoplasmic enzyme activity, depend upon the extracellular and intracellular concentration of sodium and potassium. Through these mechanisms Na^+ / K^+ - ATPase affects intracellular pH, protein synthesis, cell growth and differentiation, and calcium concentration (Smith *et al.* 1982). The restoration and maintenance of sodium and potassium concentration of cells and its extracellular spaces are maintained by sodium pump (Rodriguez *et al.* 1992). The activity of human erythrocyte membrane Na^+ / K^+ - ATPase of normal (control), capsaicin and / or capsazepine treated is given in **Table 15**.

In vitro incubation with capsaicin resulted in significant activation ($p < 0.01$) of human erythrocyte membrane Na^+ / K^+ - ATPase activity.

The concentration dependent effect of capsaicin is shown in **Figure 14.** Maximum increase in the activity of Na^+ / K^+ - ATPase is observed at 10^{-5} M capsaicin which decreased at lower concentration with almost no effect at 10^{-8} M capsaicin.

The time-dependent effect of capsaicin (10^{-6} M) on the activity of human erythrocyte membrane Na^+ / K^+ - ATPase is shown in **Figure 15.** The effect of

Figure 14



Concentration-dependent effect of capsaicin on erythrocyte membrane sodium potassium adenosine triphosphatase (Na^+/K^+-ATPase) activity

a Activity of erythrocyte membrane Na^+/K^+-ATPase is expressed μM of Pi released per hour per milligram of protein at 37° C.

* p (< 0.01) as compared to control.

Values are mean ± SD of 4 -5 independent experiments.

capsaicin was fast with significant activation of enzyme activity observed within minutes of incubation. Maximum effect was observed after 60 min of incubation, increasing the time beyond 60 min resulted in less activation and activity returned to normal level after 120 min of incubation.

In vitro capsazepine (10^{-5} M) treatment did not affect Na^+ / K^+ - ATPase activity. However, *in vitro* incubation of erythrocytes with capsaicin (10^{-5} M) along with capsazepine (10^{-5} M) caused activation of Na^+ / K^+ - ATPase activity. **Table 15.**

TABLE -15

***In vitro* effect of capsaicin and / or capsazepine on erythrocyte membrane sodium-potassium adenosine triphosphatase (Na^+/K^+-ATPase) activity.**

Conditions		n	Na^+/K^+-ATPase activity[a]
Control		13	0.040207 ± 0.00023
+ Capsaicin	(10^{-5} M)	12	0.079270 ± 0.00019*
+ Capsazepine	(10^{-5} M)	14	0.03913 ± 0.00021
+ Capsaicin and Capsazepine	(10^{-5} M) and (10^{-5} M)	11	0.06028 ± 0.00037*

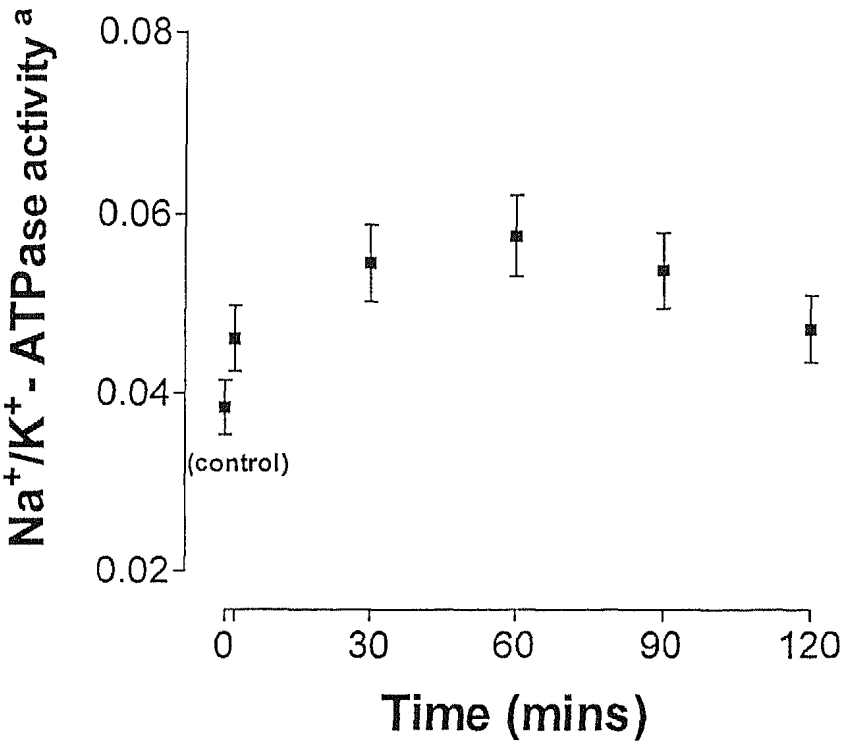
n number of subjects.

a Activity of Na^+/K^+-ATPase is expressed as µM of Pi liberated / hour / mg of protein at 37°C.

* p (<0.01) as compared to control

Values are mean ± S.D. of 4 - 5 independent experiment in each group.

Figure 15



Time-dependent effect of capsaicin on erythrocyte membrane sodium-potassium adenosine triphosphatase (Na^+/K^+-ATPase) activity

a Activity of erythrocyte membrane Na^+/K^+-ATPase is expressed as μM of Pi released per hour per milligram of protein at 37° C.

Values are mean ± SD of 4 -5 independent experiments.

No significant change after capsazepine (10^{-5} M) treatment clearly signify that this vanilloid compound did not have any significant effect on human erythrocyte Na^+ / K^+ - ATPase activity.

Na^+ / K^+- ATPase is a transmembrane enzyme primarily responsible for the active transport of sodium and potassium ions in mammalian cells. This protein is greatly expressed by neurons and consumes 30-60% of brain ATP stores, it maintains the electrical potential for the excitability of this tissue (Glynn, 1985). The activation of the human erythrocyte membrane Na^+ / K^+ - ATPase activity by capsaicin is explained on the basis of concentratoin dependent effect of capsaicin on the fluidity of the erythrocyte membrane (Meddings *et al.* 1991).

Capsaicin is a lipophilic molecule and is able to interact with the phospholipid milieu of the membrane and causes change in the ionic fluxes as a result of which the thermotropic property is affected (Skou, 1988). Moreover, change in fluidity has been found to stimulate sodium pumping activity in human erythrocytes (Giraud et al, 1980). Na^+ / K^+ - ATPase being an integral protein spans the red cell membrane, the conformational state and hence its catalytic activity may be dictated by the vicinal activating phospholipids since a direct interaction between the enzyme and phospholipids has been shown by Muczynski *et al.* (1983).

Since Na^+ / K^+ - ATPase transports $3Na^+$ outwards and $2K^+$ inwards across the plasma membrane, stimulation of Na^+ / K^+ - ATPase activity will result in hyperpolarization of the cell membrane (Kahn *et al.* 1993). This hyperpolarization could lead to capsaicin - induced vasodilaton (Aranda *et al.* 1995) an effect which may have physiological implications in humans.

5.4 *INTRACELLULAR REDUCED GLUTATHIONE CONTENT*

Glutathione (GSH), a tripeptide consisting of glutamyl -L-cysteinyl - glycine is the most abundant intracellular thiol compound present in virtually all mammalian tissues (Meister, 1976; Sen, 1997). GSH is one of the major intracellular constituent of erythrocytes, it protects erythrocytes from oxidative damages by reacting with free radicals and peroxides (Kondo *et al.* 1981) and itself gets oxidised resulting in appearance of oxidized glutathione (GSSG) in the medium surrounding the erythrocytes.

Functions of GSH in reductive processes are essential for the synthesis and also degradation of proteins, formation of the deoxyribonucleotide and regulaton of enzymes (Sen *et al.* 1994).

GSH by its multifunctional properties attracts interest in the biosynthesis of macromolecules, intermediary metabolism, drug metabolism, radiation cancer, oxygen toxicity, transport, immunology, endocrinology, environmental toxins, aging and exercise (Pesonen and Anderson, 1991).

In addition, glutathione may have clinical importance since enzyme deficiencies of the glutathione metabolism may cause hemolytic anemia and neurologic symptoms in children (Meister, 1983) and decreased glutathione levels have been reported in several diseases including AIDS, diabetes and Parkinson's disease (Gul *et al.* 2000).

GSH has other important functions in human cells, apart from its role in xenobiotic metabolism. It participates in the decomposition of potentially toxic

hydrogen peroxide in the reaction catalyzed by GSH peroxidase . GSH is an important intracellular reductant, helping to maintain essential SH groups of enzymes in their reduced states. When GSH acts as a reducing agent, its SH becomes oxidized and forms a disulphide link with other molecule of GSH. Oxidized glutathione (GSSG), in turn, can be reduced to GSH by the action of GSSG reductase, in a reaction using NADPH. NADPH is recycled by glucose-6-phosphate dehydrogenase via the pentose phosphate pathway, which is particularly important in red blood cells (Murray, 1990). The intracellular GSH concentration of normal (control), capsaicin and / or capsazepine treated human erythrocytes is given in **Table 16**.

In vitro incubation with capsaicin resulted in an increase of intracellular GSH concentration of human erythrocytes.

The concentration-dependent effect of capsaicin (10^{-5} M - 10^{-8} M) is shown in **Figure 16**. Maximum increase in intracellular GSH concentration was observed at 10^{-5} M capsaicin which decreases subsequently at lower concentration with almost no effect at 10^{-8} M capsaicin.

The time - dependent effect of capsaicin (10^{-6} M) on intracellular GSH concentration of human erythrocyte is shown in **Figure 17.**

The effect of capsaicin was fast, with significant increase in intracellular GSH concentration, observed within minutes of incubation. Maximum increase in intracellular GSH concentration was observed after 60 min of incubation. Further increase in incubation time resulted in diminished effect and concentration of intracellular GSH returned to normal level after 120 min.

In vitro treatment of erythrocytes with capsazepine (10^{-5} M) did not have any significant effect on intracellular GSH concentration. However, *in vitro* incubation of erythrocytes with capsaicin (10^{-5} M) and capsazepine (10^{-5} M) caused an increase in intracellular GSH concentration as given in **Table 16.**

TABLE - 16

***In vitro* effect of capsaicin and / or capsazepine on erythrocyte reduced glutathione (GSH) concentration.**

Conditions		n	GSH concentration[a]
Control		12	5.8757 ± 0.2909
+ Capsaicin	(10^{-5} M)	16	7.0508 ± 0.2880*
+ Capsazepine	(10^{-5} M)	11	5.7804 ± 0.1623
+ Capsaicin and Capsazepine	(10^{-5} M) and (10^{-5} M)	13	6.8397 ± 0.279*

n number of subjects.

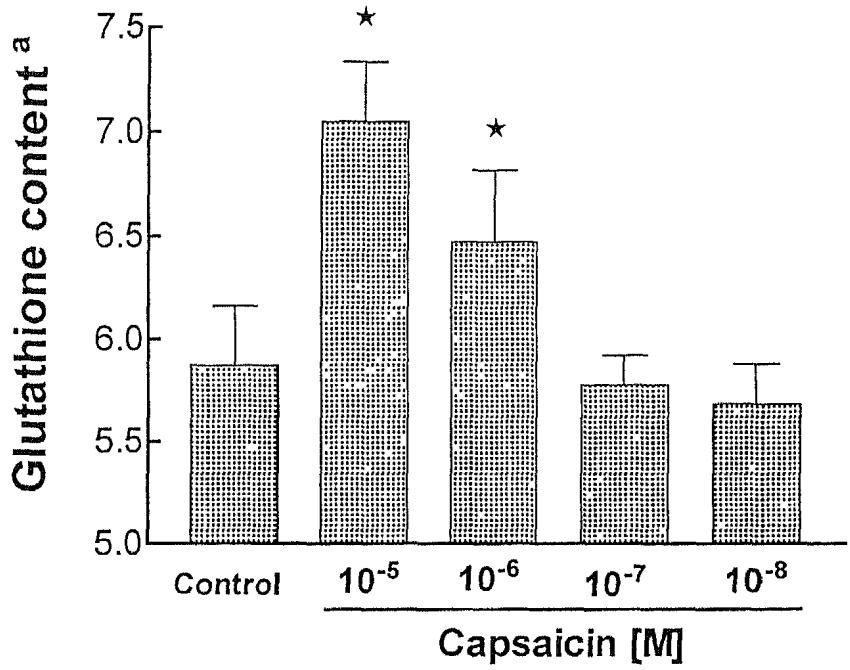
a Concentration of intracellular GSH is expressed as µM per gram of Hb at 37°C.

* p (<0.01) as compared to control

Values are mean ± S.D. of 4 - 5 independent experiment in each group.

Glutathione is an important intracellular peptide with multiple functions ranging from anti-oxidant defense to modulation of cell proliferation (Lu, 1999).

Figure 16



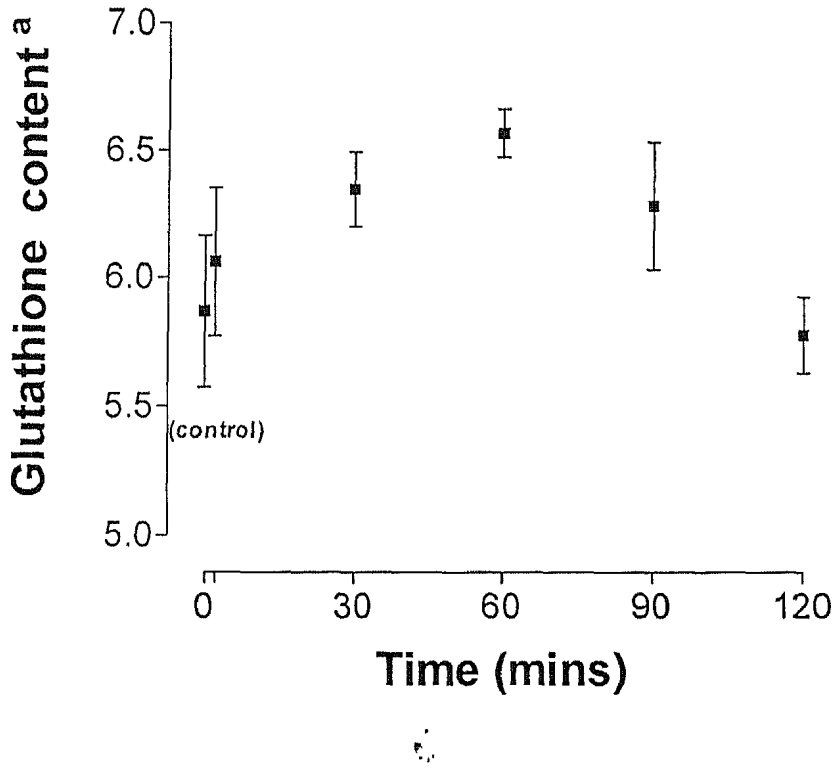
Concentration-dependent effect of capsaicin on erythrocyte intracellular reduced glutathione(GSH) content

a Concentration of erythrocyte intracellular reduced glutathione(GSH) content is expressed as μM per gram of hemoglobin at 37° C.

* p (< 0.01) as compared to control.

Values are mean ± SD of 4 -5 independent experiments.

Figure 17



Time-dependent effect of capsaicin on erythrocyte intracellular reduced glutathione(GSH) content

[a] Activity of erythrocyte intracellular reduced glutathione(GSH) content is expressed as μM per gram of hemoglobin at 37° C. Values are mean ± SD of 4 -5 independent experiments.

The increase in the intracellular GSH concentration of human erythrocytes upon *in vitro* treatment with capsaicin could be due to the effect of capsaicin on erythrocyte glucose metabolism. Capsaicin may also enhance the activity of glutathione reductase (GR), an enzyme found within the erythrocytes that reduces oxidized form of glutathione (GSSG) to its reduced form (GSH) requiring co-factor NADPH as the physiological hydrogen donor.

Our results show that capsaicin, present in capsicum has potent antioxidative capacity as evidenced by its effect on crythrocytes GSH concentration. Although, the exact mechanism by which capsaicin causes elevation in intracellular GSH concentration is not clear, our observations may, in part, explain a possible physiological benefit of high capsicum intake.

This finding may have importance in view of increasing evidence that oxidative stress and ROS induces a variety of pathological events such as atherogenesis (Steinberg *et al.* 1989), carcinogenesis (Cerrutti, 1985) and aging (Harman, 1981). Thus, dietary intake of capsicum may protect humans against the deleterious effects caused by free radicals produced from oxidative stress.

5.5 MEMBRANE SULFHYDRYL GROUPS

Sulfhydryl (SH) groups are essential in the protection against the deleterious effects of reactive oxygen species (ROS), in addition, SH groups are important for the function of many proteins. The Ca^{++} - ATPases, for example, contain an essential thiol group. Impairment of this thiol moiety leads to increased intracellular levels of Ca^{++} which frequently precedes cellular necrosis. Hormone receptors involved in maintaining Ca^{++} homeostasis often contain a critical (SH) moiety (Haenen *et al.* 1989).

To protect SH groups of proteins, high concentrations of the GSH are needed. It is difficult to estimate the level of GSH needed, since thiols may exhibit pro-oxidant and anti-oxidant activity (Rowley and Halliwelll, 1982).

The concentration of membrane SH-group of normal (control) capsaicin and / or capsazepine treated human erythrocytes is given in **Table 17.**

Effect of capsaicin and / or capsazepine on membrane SH-group was evaluated after inducing erythrocytes to oxidative stress by prior incubation with tert - butyl hydroperoxide (t-BHP). *In vitro* incubation with t-BHP (10^{-3} M - 10^{-5} M) resulted in significant ($p < 0.01$) decrease in the concentration of SH-group. Addition of capsaicin (10^{-5} M) effected a 19% protection of membrane SH-group induced by 1 mM t-BHP. The effect of capsaicin was more evident at decreasing concentrations of t-BHP.

The concentration-dependent effect of capsaicin is shown in **Figure 18.** Maximum protection of membrane SH-group was observed at 10^{-5} M capsaicin which decreases subsequently at lower concentration.

Addition of capsazepine (10^{-5} M) after inducing oxidative stress by t-BHP did not protect oxidation of membrane SH-group. However, addition of capsaicin (10^{-5} M) along with capsazepine (10^{-5} M) caused protection of membrane SH-group almost similar to that of capsaicin as given in **Table 17.**

TABLE - 17

In vitro **effect of capsaicin and / or capsazepine on erythrocyte membrane sulfhydryl (SH) groups oxidised by t-BHP.**

Conditions	n	SH -group[a]			
			t-BHP		
			10^{-3} M	10^{-4} M	10^{-5} M
Control	11	99.34±2.26	65.74±4.56*	61.57±3.71*	72.7 ± 3.74*
+ Capsaicin (10^{-5} M)	13	103.9±4 61	62.79±3.85	72.61±3.69	85.06±3.59
+ Capsazepine (10^{-5} M)	15	98 26±6.25	54.10±2.31	66.78±6.52	72.00±5.6
+ Capsaicin (10^{-5} M) and Capsazepine (10^{-5} M)	10	101.17±5.31	59.7±2.79	70.83±4.29	82.0±1.17

n number of subjects.

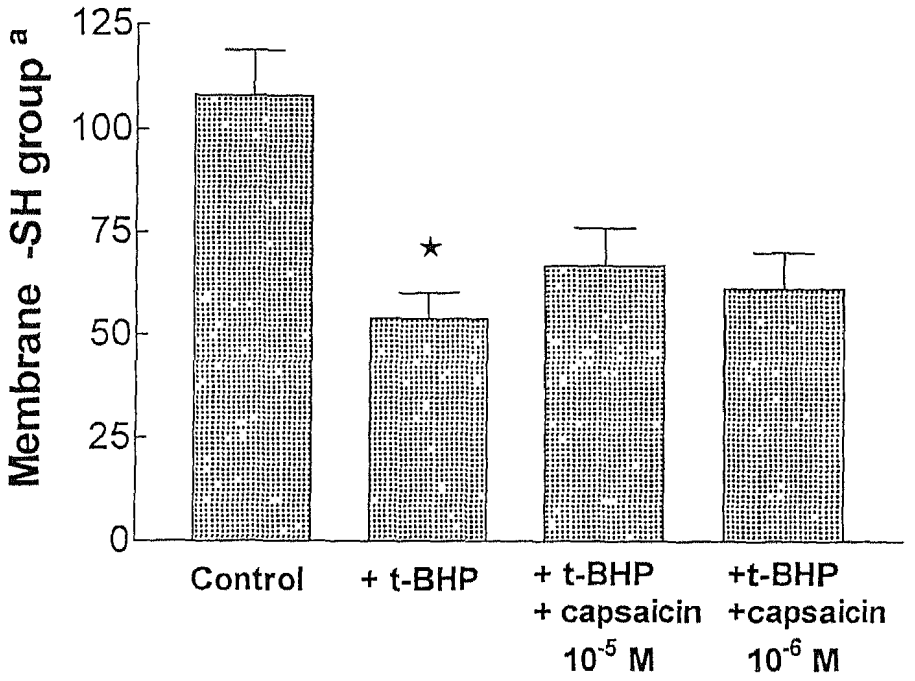
a Concentration of membrane SH-group is expressed in nM per milli gram of protein at 37°C.

* p (<0.01) as compared to control

Values are mean ± S.D. of 4 - 5 independent experiment in each group.

In recent years, the anti-oxidative properties of food constituents have been extensively investigated in an effort to characterize their efficacy as natural dietary anti-oxidants. Reactive oxygen species (ROS) mediated oxidation of biological molecules has been proposed to induce a variety of pathological events such as atherogenesis (Steinberg *et al.* 1989) carcinogenesis (Cerutti, 1985) and aging (Harman, 1981). Several spices are known to have anti-oxidative property.

Figure 18



Concentration-dependent effect of capsaicin on erythrocyte membrane sulfhydryl (-SH) groups

[a] Concentration of erythrocyte membrane sulfhydryl (-SH) groups is expressed as nM per milligram of protein at 37° C.

* p (< 0.01) as compared to control.

Values are mean ± SD of 4 -5 independent experiments.

(Bracco et al 1981). Asai *et al.* (1999) have demonstrated anti-oxidative property of capsicum extracts on phospholipid hydroperoxides in mice plasma and erythrocytes. They speculated the anti-oxidative property of capsicum due to the additive or synergistic action between the α-tocopherol and carotenoids present in capsicum extract, but there is no direct proof. Although, α-tocopherol and carotenoids may provide anti - oxidative action, our results confirm that capsaicin, present in capsicum, has potent anti oxidative capacity as evidenced by its effect on erythrocyte GSH and membrane SH-groups. Erythrocytes are exposed to higher oxygen tensions compared to other cells and is more susceptible to oxidative stress. The membrane SH-groups play an important role in the maintenance of membrane structure and functions of erythrocytes. Our observation of the protection of membrane SH-groups by capsaicin may, in part, explain a possible physiological benefit of high capsicum intake.

5.6 OSMOTIC FRAGILITY OF ERYTHROCYTES

The osmotic fragility of the erythrocytes is a property depending on the movement of water into the cells and is related to cellular deformability. The factors which determine osmotic fragility of erythrocytes are complicated and include cation content (Mentzer and Clark, 1983), cellular age (Rifkind *et al.* 1983), individual age (Araki and Rifkind, 1980), and a change in cell shape (Chan *et al.* 1975). An increased osmotic fragility is of clinical significance because it has been linked to several diseases that result in hemolytic anaemia (Mentzer and Clark, 1983).

Red cell volume is not only dependent upon the physical state of the cell's constituents but also on the cell's total solute concentration and the particular composition of the intracellular and extracellular compartments. Any mechanism

which could provide control of the total solute content could also provide for the regulation of the cell volume. In red cells, since K^+ and Na^+ represent the principal cations, control of cell volume would follow from control of the cell's sum sodium and potassium. Cell volume is constant when the rates of active Na^+ and K^+ transport equal the rates at which Na^+ and K^+ move down their electrochemical potential gradients. Volume regulation is therefore sensitive to both the pump rate and the leakage rate and any change in either parameter will have its consequences in readjusting the cell to a new steady state.

The osmotic fragility profile of normal (control), capsaicin and/or capsazepine treated human erythrocytes is shown in **Figure 19.**

The effect of capsaicin and / or capsazepine on the MEF of erythrocytes is given in **Table 18.**

Our result shows that osmotic fragility of erythrocytes is altered after *in vitro* capsaicin (10^{-5} M) incubation, however *in vitro* incubation with capsazepine (10^{-5} M) did not have any significant effect on OF and MEF. Capsaicin (10^{-5} M) along with capsazepine (10^{-5} M) did alter the OF and MEF as given in **Table 18** and shown in **Figure 19** respectively.

Capsaicin (10^{-5} M) exerts a pronounced effect on the erythrocyte osmotic fragility which is evident by a significant shift of the curve to the left and a decrease in the MEF representing a resistant to lysis.

TABLE - 18

In vitro **effect of capsaicin and / or capsazepine on human erythrocyte osmotic fragility.**

Conditions		n	MEF[a]
Control	14		0.687 ± 0.002
+ Capsaicin	(10^{-5} M)	11	0.668 ± 0.008*
+ Capsazepine	(10^{-5} M)	13	0.587 ± 0.006
+ Capsaicin (10^{-5} M) and Capsazepine	(10^{-5} M)	10	0.672 ± 0.007*

n number of subjects.

a Mean erythrocyte fragility which is NaCl concentration of 50% hemolysis of erythrocytes. .

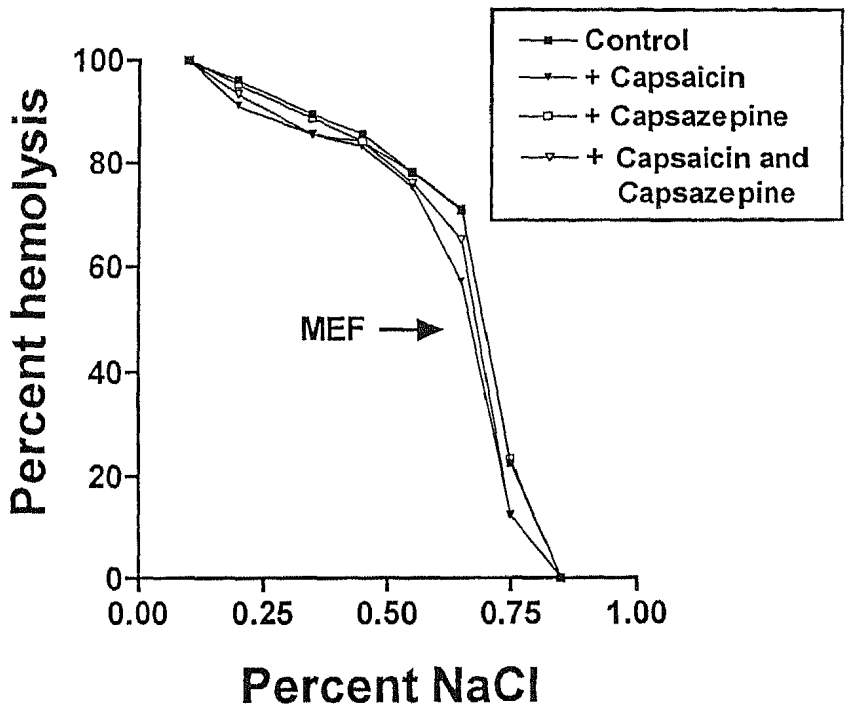
* p (<0.01) as compared to control

Values are mean ± S.D. of 4 - 5 independent experiment in each group.

A significant ($p < 0.01$) decrease in MEF, as compared with control, is observed when erythrocytes are incubated with 10^{-5} M capsaicin, however, no significant alteration is observed with lower concentrations (10^{-6} and 10^{-7} M) of capsaicin.

The present observation of the effect of capsaicin on the osmotic fragility of human erythrocytes depends on the movement of water into the cells and is a measure of erythrocyte tensile strength. An altered osmotic fragility could be due

Figure 19



Erythrocyte osmotic fragility curve of normal erythrocytes (control), capsaicin and/or capsazepine treated erythrocytes

MEF : Mean erythrocyte fragility, the NaCl concentration of 50% hemolysis of erythrocytes.
Values are mean of 4 - 5 experiments in each group.

to either perturbation in erythrocyte membrane structure or due to changes in intra- cellular metabolism (Hillman and Finch, 1992). Capsaicin is a lipophilic molecule and is reported to get incorporated in phospholipid bilayer affecting the thermotropic properties (Aranda *et al.* 1995). It has also been reported that capsaicin affects the physical properties (fluidity) of non neuronal cell plasma membrane (Meddings *et al.* 1991). Since the effect of capsaicin on erythrocyte is fast (within 30 min of incubation) and is not time dependent, it is likely that decreased MEF is due to a direct interaction of capsaicin with the erythrocyte membrane rather than due to alteration in the intracellular metabolism of erythrocytes. Although, it is difficult to speculate the exact mechanism of action of capsaicin on erythrocyte, it is obvious that the action of capsaicin is not receptor mediated since no dose responsive effect is observed.

6. *CONCLUSION*

It remains a mystery why is it that the same hot taste of capsicum which repels a variety of mammals from rats to squirrel to dogs, is found pleasurable by humans. Psychologists speculated that eating hot peppers may be a form of masochism. This theory is however at variance with the well-known geographic pattern of hot peppers consumption, namely, people living in tropical climates prefer their food hotter than those residing in temperate climates. This is due to the fact that eating hot and spicy food helps combat the warm climate via gustatory sweating. Nonetheless, the human liking or aversion towards the taste of hot peppers is probably far more complex than this simple physiological implication.

In 1997, receptor for capsaicin named vanilloid receptor (VR1) was cloned, which functions as a molecular integrator of painful chemical and physical stimuli including noxious heat and low pH. Both heat and low pH are able to activate capsaicin-gated ion-channel and this is the reason why capsaicin is "hot tasting" to humans.

Among irritant compounds acting on sensory neurons, capsaicin and its related vanilloids are unique in that the initial stimulation by vanilloids is followed by a lasting refractory state, traditionally termed desensitization. This property of capsaicin has led to its use as an analgesic agent in chronic painful disorders. Capsaicin also exerts a variety of effects on non-neuronal tissues. These actions considered non-specific include : inhibition of cardiac muscle excitability, inhibition of visceral smooth muscle activity, contraction of vascular smooth muscle, inhibition of prostanoid formation, inhibition of platelet

aggregation, influencing a variety of enzyme activities, stimulation of the migration of human polymorphonuclear cells and blocking melanograph cells in rat pituitary. These non-specific effects are sustained and do not undergo desensitization and are easily reproducible on reapplication of capsaicin, whereas the sensory neuron selective effects are produced by nanomolar (>10 nM) concentration of capsaicin, however, it is typically micromolar (>3 μM) concentration of capsaicin that are required to cause its cell non-selective effects. The biological relevance and the molecular mechanism of capsaicin's effect remains unclear.

Very few studies has been done to explore the effect of capsaicin on erythrocytes. Our observations show that capsaicin induces a variety of alterations in red cell enzyme activities and concentration of intracellular metabolites. Capsaicin exerts a stabilizing effect on erythrocytes making them more resistant to lysis under hypotonic stress. Capsaicin - induced transient activation of erythrocyte AChE, Ca^{++} - ATPase and Na^{+} / K^{+} - ATPase may have important physiological implications in the maintenance of ionic balance in the cells, in establishing the negative electrical potential inside the cells and in facilitating a number of specialized functions such as cell volume, osmotic pressure, excitability and muscle contraction. The transient increase in the concentration of erythrocyte metabolite i.e. GSH and protection of membrane SH-groups by capsaicin are also significant findings. These effects are due to potent anti-oxidant capacity of capsaicin.

It is difficult on the basis of present observation to explain the exact mechanism of action of capsaicin on non-neuronal tissues leading to a wide range

of effects. However, some of the effects of capsaicin on erythrocytes may be explained by the following hypothesis.

1. Capsaicin interacts with lipid bilayer of erythrocyte membrane resulting in alteration of membrane fluidity and perturbation of membrane structure.

2. Alteration in membrane fluidity causes change in the activity of membrane bound enzymes.

3. The interaction of capsaicin with the plasma membrane is dependent on the concentration of capsaicin in blood. Thus the effect of capsaicin decreases as soon as the level of capsaicin goes down in blood.

4. Capsaicin-induced activation of ion-channels (Na^+, K^+, Ca^{++}) cause initiation of nerve impulses by depolarising the membrane potential and also raise the level of intracellular ions.

5. The anti-oxidative property enables capsaicin to act as scavengers of free radicals and prevents oxidative stress.

On the basis of present observations, it is reasonable to assume that high consumption of chillies by human population in certain regions of the world (especially hot climatic regions) may confer certain physiological benefits and may provide a mechanism for adaptation in hot climates.

7. REFERENCES

ABDEL SALAM OME, SZOLCSANYI J, BARTHO L & MOZSIK GY 1994. Sensory nerve-mediated mechanisms, gastric mucosal damage and its protection. A Critical Overview. *Gastroprotection* **2** : 4 - 12.

ACS G, PALKOVITS M & BLUMBERG PM 1994. Comparison of ^{3}H resiniferatoxin binding by the vanilloid (capsaicin) receptor in dorsal root ganglia, spinal cord, dorsal vagal complex, sciatic and vagal nerve and urinary bladder of the rat. *Life Sci.* **55** : 1017 - 1026.

AGRAWAL RC, WIESSLER M, HECKER E & BHIDE SV 1986. Tumour-promoting effect of chilli extract in BALB/C Mice. *Int. J. Cancer* **38** : 689 - 695.

ALLAN D, WATTS R & MICHELL RH 1976. *Biochem. J* **156** : 225.

AMANN R & MAGGI CA 1991. Ruthenium red as a capsaicin antagonist. *Life Sci.* **49** : 849 - 856.

ANDERSON RA & MARCHESI VT 1985. Regulation of the association of the membrane skeletal protein - 4.1 with the glycophorin by a polyphosphoionositide. *Nature* **318** : 295 - 298.

APPENDINO G & SZALLASI A 1997. Euphorbium : modern research on its active principle resiniferatioxin, revives an ancient medicine. *Life Sci.* **60** (10) : 681 - 696.

ARAKI K & RIFKIND JM 1980. *J. Gerontol* **35** : 499.

ARANDA FJ, VILLALAIN J & GOMEZ-FERNANDEZ JC 1995. *Biochim. Biophys. Acta.* **1234** : 225.

BALLAS SK, KLIMAN HJ AND SMITH ED 1985. Glyceraldehyde-3-phosphate dehydrogenase of rat erythrocyte has no membrane component. *Biochim. Biophys. Acta,* **831 :** 142 - 149.

BAUMAN TK, BURCHIEL KJ, INGRAM SL & MARTENSON ME 1996. Responses of adult human dorsal root ganglion neurons in culture to capsaicin and low pH. Pain. **65 :** 31 - 38.

BEUTLER E 1984. Red Cell Metabolism : A Manual of Biochemical Methods (Grunne and Stratton, New York).

BEVAN S & DOCHERTY RJ 1993. Cellular mechanisms of the action of capsaicin, in capsaicin in the study of pain (Wood JN ed) pp. 27-44. *(Academic Press San Diego, CA).*

BEVAN S & SZOLCSANYI J 1990. Sensory neuron specific actions of capsaicin mechanisms and applications. *Trends Pharmacol Sci.* **11** : 330 - 333.

BEVAN S & YEATS J 1991. Proton activate a cation conductance in a sub-population of rat dorsal root ganglion neurones. *J. Physiol* (London) **433 :** 145 - 161.

BEVAN S, HOTHI S, HUGHES G, JAMES IF, RANG HP SHAH K WALPOLE CSJ & YEATS JC 1992. Capsazepine : a competitive antagonist of the sensory neuron excitant by capsaicin. *Br. J. Pharmacol.* **107** : 544 - 552.

BLAUSTEIN MP 1974. *Rev. Physiol. Biochem. Pharmacol.* **70 :** 33.

BLOCH KE 1983. *CRC Critical Rev. Biochem.* **14 :** 47 - 92.

BRAND LM, SKARE KL, LOOMANS ME, RELLER HH, SCHWEN RJ, CHIABRANDO C, CASTELLI MG & TAI HH 1990. Anti-inflammatory pharmacology and mechanism of the orally active capsaicin analogs, NE - 19550 and NE-28345. *Agents Actions* **31** : 329 - 340.

BUCK SH & BURKS TF 1986. The neuropharmacology of capsaicin : A review of some recent observations. *Pharmacol. Rev.* **38** : 179 - 226.

BUCK SH, MILLER MS & BURKS TF 1982. Depletion of primary afferent substance P by capsaicin and dihydrocapsaicin without altered thermal sensitivity in rats. *Brain Res.* **233** : 216 - 220.

CAMEROON- SMITH D, COLQUHOM EQ, YE JM, HETTIAVACHCHI M & CLARK MG 1990. Capsaicin and dihydrocapsaicin stimulate oxygen consumption in the perfused rat hindlimb. *Int. J. Obes.* **44** : 611 - 616.

CAMPBELL E, BEVAN S & DRAY A 1993. Clinical applications of capsaicin and its analogues. In : Wood JN (ed), Capsaicin in the study of pain, pp. 255 - 272. (*Academic Press,* New York).

CARAFOLI E 1992. The Ca^{++}- Pump of the Plasma membrane. *J. Biol. Chem.* **267** (4) : 2115 - 2118.

CARAFOLI, E 1987. *Ann. Rev. Biochem.* **56** : 395.

CARAWAY KL, TRIPLETT RB & ANDERSON DR 1971. *Biochim. Biophys. Acta.* **379** : 571.

CATERINA MJ, SCHUMACHER MA, TOMINAGA M, ROSEN TA, LEVINE JD & JULIUS D 1997. The capsaicin receptor : A heat-activated ion-channel in the pain pathway. *Nature* **389** : 816 - 824.

CHAN TK, LACELL PL & WEED RI 1975. *J. Cell. Physiol.* **85** : 47.

CHAPMAN D & BRYNE P & SHIPLEY GG 1966. *Proc. R. Soc.* (London) Sec. A **290** : 115 - 142.

CHAPMAN D & PENKETT SA 1966. *Nature* (London) **211** : 1304 - 1305.

CHAPMAN D & QUINN PJ 1976. *Proc.Natl. Aca. Sci. USA* **73** : 3971-3975.

CHAPMAN D & SALSBURY NK 1966. *Trans. Faraday Soc.* **62** : 2607-2621.

CHAPMAN D, WILLIAMS RM & LADBROOKE ID 1967. *Chem. Phys. Lipids* **1** : 445 - 475.

CHASES JA, MOHANDAS N & SHOHET SB 1985. Erythrocyte membrane rigidity induced by glycophorin A-ligand interaction : Evidence for a ligand induced association between glycophorin A and skeletal proteins. *J. Clin. Invest.* **75** : 1919 - 1926.

COLLIER JG & FULLER RW 1984. Capsaicin inhalation in man and the uptake of sodium cromoglycate. *Br. J. Pharmacol.* **81** : 113 - 117.

CROALL DE, MORROW JS & DE MARTINO GN 1986. *Biochim. Biophys. Acta.* **882** : 287.

DACIE JV & LEWIS SM 1984. Practical Hematology pp. 152. (*Orient Longman,* UK).

DANIEL EE, NAGAHMA M, SATO H, JURY J & BOWKER P 1987. Chemical studies on substance P release from muscularis mucosa of esophagus, In substance P and neurokinins ed. by J L Henry, AC Cuello, G Pelletier, R Duirion and D Regoli pp. 266 - 269, (*Springer*-New York).

DAVSON H & DANIELLI JF 1943. In: The Permeability of Natural Membranes. (*Cambridge University Press,* Cambridge).

DRAY A 1992. Neuropharmacological mechanisms of capsaicin and related substances. *Biochem. Pharmacol.* **44** : 611 - 615.

DRAY A, BETTANEY J, RUEFF A, WALPOLE C & WRIGGLES WORTH R 1990. Antinociceptive capsaicin analogues : Studies on nociceptive fibres of the neonatal rat tail in vitro. NE - 19550 & NE-21610. *Eur. J. Pharmacol* **181** : 289 - 298.

DRAY A, CAMPBELL EA, HUGHES GA, PATEL IA, PERKINS MN, REED HP, RUEFF A, SENO N, URBAN L & WALPOLE CSJ 1991. Antagonism of capsaicin - induced activation of C-fibres by a selective capsaicin antagonist capsazepine. *Br. J. Pharmacol.* **102** : 78.

DRAY A, FORBES CA & BURGESS GM 1980. *Neurosci. Lett.* **110** : 52 - 59.

EDVINSSON L, JANSEN I, KINGMAN TA & MC CULLOCH J. 1990. Cerebro-vascular responses to capsaicin invitro and in situ *Br. J. Pharmacol.* **100** : 312 - 313.

ELLIS JL, SHAM JSK & UNDEM BJ 1994. Tachykinin independent effects of capsaicin on smooth muscle in human isolated bronchi. *Am. J. Respir. Crit Care. Med.* **155** : 751 - 755.

ELLMAN GL, COURTNEY KD, ANDRES V JR & FEATHERSTONE RM 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **7** : 88 - 95.

ENYEDI A, VORHERR T, JAMES P, McCORMICK DJ, FIOTEO AG, CARAFOLI E & PENNISTON JT 1989. *J. Biol. Chem.* **264** : 12313 - 12321.

FAIRBANKS G 1980. In : Red Cell and Lens Metabolism Eds, Srivastava SK pp. 191. (*Elsevier Inc.* North-Holland).

FISKE CH & SUBBAROW Y 1925. The colorimetric determination of Phosphorus *J. Biol. Chem.* **66** : 375 - 400.

FOX AJ, URBAN L, BARNES PJ & DRAY A 1995. Effects of capsazepine against

capsaicin- and proton provoked excitation of single airway C-fibers and vagus nerve from the guinea pig. *Neuroscience* **67** : 741 - 752.

FRANCO-CERECEDA A & LUNDBERG JM 1992. Capsazepine inhibits low pH and lactic acid evoked release of calcitonin gene-related peptide from sensory nerves in guinea pig heart. *Eur. J. Pharmacol.* **221** : 183 - 186.

FRANCO-CERECEDA A, LUNDBERG JM, SARIA A, SCHREIBMAYER W & TRITTHART HA 1988. Calcitonin gene-related peptide : Release by capsaicin and prolongation of the action potential in the guinea pig heart. *Acta Physiol Scand.* **132** : 181 - 190.

FUSCO BM, GEPPETTI P, FANCIULLACCI M & SICUTERI F 1991. Local application of capsaicin for the treatment of cluster headache and idiopathic trigeminal neuralgia. Cephalalgia **11** : 234 - 235.

GEISTHOVEL E. LUDWIG O & SIMON E 1986. Capsaicin fails to produce disturbances of autonomic heat and cold defense in an avian species (Anas platyrhynchos) Pfhigers Arch. **406** : 343 - 350.

GLINSUKON T, STITMUNNAITHUM V, TOSKULLEAO C, BURANAWUTI T & TANG KRISLANAVINONT V. 1980. Acute toxicity of capsaicin in several animal species. *Toxicon* **18** : 215 - 220.

GLYNN IM & KARLISH JD 1975. *Ann Rev. Physiol* **37** : 13 - 58.

GONZALEZ R, DUNKEL R, KOLEZTKO B, SCHUSOLZIARRA V & ALLESCHER HD 1998. Effects of capsaicin - containing red pepper sauce suspension on upper gastrointestinal motility in healthy volunteers. *Dig. Dis. Sci.* **43** : 1165 - 1171.

GOPINATH RM & VINCENZI FF 1977. *Biochim. Biophys. Res. Commun.* 77 : 1203 - 1209.

GORTER E & GRENDEL F 1925. *J. Exp. Med.* **41** : 439.

GOVINDRAJAN VS 1985. Capsicum — A Critical Review - *Food Sci. & Nutr.* **22** (2) : 160 - 176.

GREEN BG 1989. Capsaicin sentitization and desensitization on the tongue produced by brief exposures to a low concentration. *Neurosci. Lett.* **107** : 173 - 178.

GUL M, KUTAY FZ, TEMOCIN S & HANNINEN O 2000. Celllar and Clinical implications of glutathione. *Indian J. Biochem. Biophys* **38** : 625 - 634.

HAENEN GRMM, VERMEULEN NPE, TIMMERMAN H & BAST A 1989. Effect of thiols on lipid peroxidation in rat liver microsomes. *Chem. Biol. Interact.* **71** : 201 - 212.

HARRIS JR 1988. Erythrocyte Cylindrin : possible identity with ubiquitous 20S high molecular weight protease complex and prosome particle. *Indian J. Biochem. Biophys.* **25** : 459 - 466.

HARRIS JR 1988. *Indian. J. Biochem. Biophys.* **25** : 459 - 466.

HARTUNG M, LEACH J & ZIMMERMAN M 1989. The excitation of cutaneous nerve endings in a neuroma by capsaicin. *Brain Res.* **499** : 363 - 366.

HENRY CJK & EMERY B 1986. Effect of spiced food on metabolic rate. *Hum. Nutr. Clin. Nutr.* **40** : 165 - 168.

HILLMAN RS & FINCH CA 1992. Red Cell Manual pp. 112. (*FA, Davis Company. Philadelphia*).

HOLZER P 1991. Capsaicin : cellular targets, mechanisms of action, and selectivity for thin sensory neurons. *Pharmacol. Rev.* **43** : 144 - 201.

HOLZER P, BARTHO L, MATUSAK O & BAUER V 1990. Calcitonin-gene related peptide action on intestinal circular muscle. *Am. J. Physiol.* **256** : G 546 - G 552.

JAIN SK & SHOHET SB 1981. *Biochim. Biophys. Acta.* **642** : 46.

JAMES IF, WALPOLE CSJ, WOOD JN & WRIGGLESWORTH R 1988. Long-lasting agonist activity produced by a capsaicin-like photoaffinity probe. *Mol. Pharmacol.* **33** : 643 - 649.

JAMES PH, MAEDA M, FISCHER R, VERMA AK, KREBS J, PENNISTON JT & CARAFOLI E 1988. *J. Biol. Chem.* **263** : 2905 - 2910.

JANCSO N & JANCSO-GABOR A 1949. Desensitisation of sensory nerve endings (in Hungarian). *Kiserletes Orvostudomany (Suppl.)* **2** : 15.

JOHNSTON DS, SANGHERA S, PONS M & CHAPMAN D 1980. *Biochim. Biophys. Acta* **602** : 57 - 69.

JONES NL, SHABIB S & SHERMAN PM 1997. Capsaicin as an inhibitor of the growth of the gastric pathogen *Helicobacter pylori*. *FEMS Microbiol. Lett* **146** (2) : 223 - 227.

KAHN RH & GILMAN AG 1984. *J. Biol. Chem.* **259** : 6235.

KAMADA T, MCMILLAN DE, YAMASHITA T & OTSUJI S 1992. *Diabetes Res. Clin Pract.* **16** : 1 - 6.

KARAWYA MS *et al.* 1967. A micromethod for the determination of capsaicin in capsicum fruits. *Analyst.* **92** : 581 - 583.

KARLSSON JA 1996. The role of capsaicin - sensitive -C-fibres afferent nerves in the cough reflex. *Pulm. Pharmacol.* **9** : 315 - 321.

KAY MMB 1984. *Proc. Natl. Acad. Sci. 31* : 5753.

KENNINS P. 1982. Responses of single nerve fibres to capsaicin applied to the skin. *Neurosci. Lett.* **29** : 83 - 88.

KESSLER F, BENNARDINI F, BACHS O, SERRATOSA J, JAMES P, CARIDE AJ, GAZOTTI P, PENNISTON JT & CARAFOLI E 1990. *J. Biol. Chem* **265** : 16012 - 16019.

KITAJIMA H, YAMAGUCHI T & KIMOTO E 1990. *Biochem. J.* **108** : 1057 - 1062.

KOCHCHAR SL 1995. Economic botany in tropics; spices, condiments and other flavouring. IIIrd *Rep.*pp. 259-260 (*Macmillan India Ltd.*, New Delhi).

KRESS M, VYKLICKY L & REEH PW 1996. The capsaicin-receptor : Target for a novel action of aspirin like drugs ? *Soc. Neurosci. Abstr.* **22** : 409 I.

LADBROOKE BD, WILLIAMS RM & CHAPMAN D 1968.*Biochim. Biophys. Acta.* **150** : 333 - 340.

LANG E, NOVAK A, REEH PW & HANDWORKER HO 1990. Chemosensitization of fine afferents from rat skin invitro. *J. Neurophysiol* **63** : 887 - 901.

LEMBECK F 1987. Columbus and capsaicin : past, present and future. *Acta Physiol. Hung.* **69** : 265 - 273.

LEVY J, SOWERS JR & ZEMEL MB 1990. Abnormal Ca^{++}-ATPase activity in erythrocytes of Non-insulin-dependent diabetic rats. *Horm. metabol. Res.* **22** : 136 - 140.

LIM K, YOSHIOKA M, KIKUZATO S, KIYONAGA A, TANAKA H, SHINDO M & SUZUKI M 1997. Dietary red pepper ingestion increases carbohydrate oxidation at rest and during exercise in runners. *Med. Sci. Sports. Exerc.* **29** : 355 - 361.

LIU L & SIMON SA 1994. A rapid capsaicin-activated current in rat trigeminal ganglion neurons. *Proc. Natl. Acad. Sci. USA.* **91** : 738 - 741.

LIU L & SIMON SA 1996. Similarities and differences in the currents activated by capsaicin, piperine and zingerone in rat trigeminal ganglion cells. *J. Neurophysiol.* **76** : 1858 - 1869.

LIU L, LO Y-C, CHEN I-J & SIMON SA 1997. The responses of rat trigeminal ganglion neurons to capsaicin and two non-pungent vanilloid receptor agonists, olvanil and glyceryl nonamide. *J. Neuro. Sci.* **17** : 4101 - 4111.

LIU L, SZALLASI A & SIMON SA 1998. A non pungent resiniferatoxin analogue, phorbol 12-phenylacetate 13-acetate 20- homovanillate, reveals vanilloid receptor subtypes on rat trigeminal ganglion neurons. *Neuroscience* **84** : 569 - 581.

LOPEZ- CARILLO L, ARILA MH AND DUBROW R 1994. Chili pepper consumption and gastric cancer in Mexico : A Case Control study *Am. J. Epidemiol.* **139** : 263 - 271.

LOU YP & LUNDBERG JM 1992. Inhibition of low pH-evoked activation of airway sensory nerves by capsazepine, a novel capsaicin-receptor antagonist. *Biochem. Biophys. Res. Comm.* **189** : 537 - 544.

LOW PS, WAUGH SM, ZINKE K & DRENKHALM D 1985. Science **227** : 531.

LOWRY OH, ROSENBROUGH NJ, FARR AL & RANDALL RJ 1951.Protein measurement with the Folin-Phenol reagent. *J. Biol. Chem. 193* : 265 - 275.

LU SC 1999. Regulation of hepatic gluttathione synthesis: Current concepts and controversies. *FASEB J.* **13** : 1169 - 1183.

LUKACOVIC MF, TOON MR & SOLOMON AK 1984. Site of red cell cation leak induced by mercurial sulfhydryl reagents. *Biochimica et Biophysica Acta.* **772** : 313 - 320.

LUNDBERG JM 1993. Capsaicin-sensitive sensory nerves in the airways-implications for protective reflexes and disease in capsaicin in the study of Pain (Wood JN ed) pp. 219 - 238. (*Academic Press,* San Diego, CA).

LUTZ HU 1981. *Acta Biol. Med. Gen* **440** : 393.

MACHO A, CALZADO MA, MUNOZ-BLANCO J, GOMEZ-DIAZ C, MOLLINEDO F, NAVAS P & MUNOZ E 1999. Selective induction of apoptosis by capsaicin in transformed cells : the role of reactive oxygen species and calcium. *Cell Death Differ.* **6 (2)** : 155 - 165.

MAGGI CA 1991. The Pharmacology of the efferent function of sensory nerves. *J. Auton. Pharmacol.* **11** : 173 - 208.

MAGGI CA, & MELI A 1988. The sensory efferent function of capsaicin sensitive sensory neurons. *Gen. Pharmacol.* **19** : 1 - 43.

MAGGI CA, BORSINI F, SANTICIOLI P, GEPPETTI P, ABELLI L, EVANGELISTA S, MANZINI S, THEODORSSON - NORHEIM E., SOMMA V, AMENTA F, BACCIARELLI C & MELI A 1987. Cutaneous lesions in capsaicin treated rats. A trophic role of capsaicin - sensitive afferents ? *Nauyn-Schmiedeberg's Arch. Pharmacol* . **336** : 538 - 545.

MAGGI CA, PATTACCHINI R, QUARTARA L, ROVERO P AND SANTICIOLI P 1991. Tachykinin receptors in the guinea pig isolated bronchi. *Eur. J. Pharmacol.* **197** : 167 - 175.

MARCHESI VT & PALADE VE 1967. The localization of Mg-Na-K activated adenosine triphosphatase on red cell ghost membranes. *J. Cell. Biol.* **35** : 385 - 404.

MARSH SH, STANSFELD CE, BROWN DA, DAVEY R & McCARTHY D 1987. The mechanism of action of capaicin on sensory C-type neurons and their axons in vitro. *Neuroscience* .**23** : 275 - 290.

MARTENSON ME, INGRAM SL & BAUMANN TK 1994. Potentiation of rabbit trigeminal responses to capsaicin in a low pH environment. *Brain Res.* **651** : 143 -147.

MASSOULIC J & BONN S 1982. *Annu, Rev. Neurosci.* **5** : 57.

MATHUR R, DANGI RS, DASS SC, & MALHOTRA RC 2000. The hottest chilli variety in India, *Curr. Sci.* **79 (3)** : 287 - 288.

MATUCCI-CERINIC M, MARABINI S, JANTSCH S, CAGNONI M & PARTSCH G 1990. Effects of capsaicin on the metabolism of rheumatoid arthritis synoviocytes *in vitro*. *Ann. Rheum. Dis.* **49** : 598 - 602.

MEDDINGS JB, HOGABOAM CM, TRAN K, REYNOLDS JD & WALLACE JL 1991. Capsaicin effects on non-neuronal plasma membranes. *Biochim. Biophys. Acta.* **1070** : 43 - 50.

MEISTER A & ANDERSON ME 1983. Glutathione. *Ann. Rev. Biochem.* **52** : 711.

MEISTER A 1976. Glutathione and the γ-glutamyl cycle, in Glutathione : metabolism and function ed. by IM Anas & WB Jakoby.pp. 35. (*Raven Press*, New York).

MENTZER WC & CLARK MR 1983 in Biomembranes : Pathological membranes. Ed. Novotny *A* Vol. 2 pp. 79. (*Plenum* New York).

MILLER MS, BUCK SH, SIPES IG & BURKS TF 1982. Capsaicinoid-induced local and systemic anti-nociception without substance P depletion. *Brain Res.* **224** : 193 - 197.

MOORE DJ, CHUHEH PJ & MOORE DM 1995. Capsaicin inhibits preferentially the NADH oxidase and growth of transformed cells in culture. *Proc. Natl. Acad. Sci.* USA **92** : 1831 - 1835.

MORARIU VV, CHIS AM & ZNANIROVSCHI V 1996. *Cytobios.* **86** : 53 - 64.

MORITOKI H, TAKASE H & TANIOKA A 1990. Dual effects of capsaicin on responses of the rabbit ear artery to field stimulation. *Br J. Pharmacol.* **99** : 152 - 156.

MURRAY RK in Harper's biochemistry edited by RK Murray, DK Granner. PA Mayes & VW Rodwell 1990. pp. 645. (*Prentice - Hall, International Inc.* Connecticut)

NADKARNI AK 1976. Indian Materia Medica. Vol. 1. pp 269. (*Popular Prakashan Pvt. Ltd.* Bombay, India).

NELSON GJ 1992. In "Blood Lipids : Quantitation Composition and Metabolism". G.J. Nelson ed. pp.- 371-386. (*Wiley lotev Science,* New York).

NIGGLI V, PENNISTON JT & CARAFOLI E 1979. *J. Biol Chem.* **254** : 9955 - 9958.

NOTANI PN & JAYANT DK 1987. Role of diet in upper aerodigestive tract cancers. *Nutr. Cancer* **10** : 103 - 113.

OTT P 1985. Membrane acteylcholinesterases : Purification, molecular properties and interaction with amphiphillic environments. *Biochim. Biophys. Acta.* **822** : 375 - 392.

PALEK J, STEWART G & LIONETTI FJ 1974. *Blood.* **44** : 583.

PATTACCHINI R, MAGGI CA & MELI A 1990. Capsaicin like activity of natural pungent substances on peripheral endings of visceral primary afferent neurons. *Nauyn Schelmeidebergs Arch. Pharmacol.* **342** : 72 - 77.

PATTACCHINI R, SANTICIOLI P, GIULIANI S, MAGGI CA & MELI A 1988. Cadmium chloride induces contractions of the rat isolated urinary bladder by activation of capsaicin- sensitive sensory nerves. *Eur. J. Pharmacol.* **148** : 449 - 452.

PENKETT SA, FLOOK AG & CHAPMAN D 1968. *Chem. Phys. Lipids* **2** : 273 - 290.

PERKINS MN & CAMPBELL EA 1992. Capsazepine reversal of the anti-nociceptive action of capsaicin invivo. *Br. J. Pharmacol.* **107** : 329 - 333.

PESONEN M & ANDERSON T 1991. Characterization and induction of xenobiotic metabolizing enzyme activities in a primary culture of rainbow trout hepatocytes. *Xenobiotica* **21** : 461.

PETERSEN M & LAMOTTE RH 1993. Effects of protons on the inward current evoked by capsaicin in isolated dorsal root ganglion cells. *Pain* **54** : 37 - 42.

PRALL YG, GAMBHIR KK & AMPY FR 1998. *Life Sci.* **63** : 177 - 184.

PRUTHI JS 1987. Spices and Condiments IIIrd edn. National Book Trust. (*Aruna Press*, India).

RAEES BU & VINCENZI FF 1980. *J. Pharm. Math* **4** : 273.

RESTALL C, ARONDO JLR, ELLIOT DA, JASKOWSKA A, WEBER WV & CHAPMAN D 1981. *Biochim. Biophys. Acta* **670** : 433 - 440.

RIFIKIND JM, ARAKI K & HADLEY EC 1983. *Arch. Biochem. Biophys.* **222** : 582.

ROBERTSON JD 1959. *Biochem. Soc. Symp.* **16** : 3.

RODRIGUEL, DELORES & ARNAIS G 1992. *Mol. Neurobiol.* 6(4) : 359.

ROSENBARY SA & GUIDOTTI G 1968. *J. Biol. Chem.* **243** : 1985.

ROWLEY DA & HALLIWELL B 1982. Superoxide-dependent formation of hydroxyl radicals in the presence of thiol compounds. *FEBS lett.* **138** : 33 - 36.

RUMENJAK V 1998. *Acta. Med. Croatica* **52** : 187 - 189.

RUMSFIELD JA & WEST DP 1991. Topical capsaicin in dermatologic and peripheral pain disorders. *DICP Ann. Pharmacother.* **25** : 381 - 387.

SANN H, HARTI G, PIERAU F-K & SIMON E 1987. Effect of capsaicin upon efferent and efferent mechanisms of nociception and temperature regulation in birds. *Can J. Physiol. Pharmacol.* **65** : 1347 - 1354.

SANTICIOLI P, DEL BIANCO E, FIGINI M, BEVAN S & MAGGI CA 1993. Effect of capsazepine on the release of calcitonin gene-related peptide-like (CGRP-LI) immunoreactivity induced by low pH, capsaicin and potassium in rat soleus muscle. *Br J. Pharmacol.* **110** : 609 - 612.

SANTICIOLI P, PATACCHINI R, MAGGI CA & MELI A 1987. Exposure to calcium-free medium protects sensory fibers from capsaicin desensitization. *Neurosci. Lett.* **80** : 167 - 172.

SASAKI H, SEKIZAWA K, YANAI M, ARAI H, YAMAYA M & OHRUI T 1997. New strategies for aspiration pneumonia. *Intern. Med.* **36** : 851 - 855.

SAUBERMAN N, FORTIER NL, FAIRBANKS G, O'CONNOR RJ & SYNDER LN 1979. *Biochim. Biophys. Acta.* **559** : 292 - 313.

SCHATZMANN HJ 1983. The red cell calcium pump. *Ann. Rev. Physiol.* **45** : 303 - 313.

SCHWARTZ SM, BOSTWICK HE & MODOW MM 1972. *Curr. Top. Membr. Transp.* **3** : 1

SEN CK & HANNINEN O 1994. Physiological antioxidants in Exercise and oxygen toxicity ed. by CK Sen, L Packer & O Hanni nen pp. 89. (*Elsevier. Amsterdam*).

SEN CK 1997. Nutritional biochemistry of cellular glutathione. *J. Nutr. Biochem.* **8** : 660.

SHEETZ M & SAWYER D 1978. *J. Supramol. Struct.* **8** : 399.

SHEETZ MP & SINGER SJ 1977. *J. Cell. Biol.* **73** : 638.

SINGER SJ & NICHOLSON GL 1972. The fluid mosaic model of structure of cell membranes. *Science 175*: : 720-731.

SIZER F & HARRIS N 1985. The influence of common food additive and temperature on threshold perception of capsaicin. *Chem. Senses.* **10** : 279 - 286.

SMITH RL, MACARA IG, LEVENSON R, HOUSRAM & CANTLEY L 1982. *J. Biol. Chem.* **257** : 773.

SORENSEN K, GENTINETTA R & BRODBECK U 1982. An amphiphile-dependent form of human brain caudate nucleus acetylcholinesterase : purification and properties. *J. Neurochem.* **39** : 1050-1060.

SRINIVASAN MR & SATYANARAYANA MN 1989. Effect of capsaicin on lipoprotein lipase in rats fed high fat diet. *Indian J. Exp. Biol.* **27** : 910 - 912.

SRIVASTAVA SK & BEUTLER E 1969. *Biochem. Biophys. Res. Comm.* **28** : 659.

STAUFENBIEL M & LAZARIDES E 1986. Ankyrin is fatty acid acylated in erythrocytes. *Proct. Natl. Acad. Sci. (USA)* ***83***: : 318 - 322.

STIEGER J & LUTERBACHER S 1981. *Biochim. Biophys. Acta* **641** : 270 - 275.

STJARNE P, LUNDBLAD L, LUNDBERG JM & ANGGARD A 1989. Capsaicin and nicotine sensitive afferent neurons and nasal secretions in healthy human volunteers and in patients with vasomotor rhinitis. *Br. J. Pharmacol.* **96** : 693 - 701.

SUHAIL M & RIZVI SI 1987. Red cell membrane (Na^+/K^+)-ATPase in diabetes mellitus. *Biochem. Biophys. Res. Comm.* **146 (1)** : 179 - 186.

SURH Y-J & LEE SS 1995. Capsaicin a double edged sword : Toxicity, metabolism and chemopreventive potential. *Life Sci.* **56** : 1845 - 1855.

SZALLASI A & BLUMBERG PM & LUNDBERG JM 1995. Proton inhibition of [^{3}H] Resiniferatoxin binding to vanilloid (capsaicin) receptors in rat spinal cord. *Eur. J. Pharmacol.* (Mol. Pharmacol. Sect.) **289** : 181 - 187.

SZALLASI A & BLUMBERG PM 1990a. Specific biding of resiniferatoxin, an ultrapotent capsaicin analogue, by dorsal root ganglion membranes. *Brain Res.* **524** : 106 - 111.

SZALLASI A & BLUMBERG PM 1990b. Resiniferatoxin and its analogues provide novel insights into the pharmacology of the vanilloid (capsaicin) receptor. *Life Sci.* **47** : 1399 - 1408.

SZALLASI A & BLUMBERG PM 1993. Mechanisms and therapeutic potential of vanilloids (capsaicin-like molecules). *Adv. Pharmacol. 24*: : 123 - 155.

SZALLASI A & BLUMBERG PM 1999. Vanilloid (Capsaicin) receptors and mechanisms. *Pharmacol Rev.* **51 (2)** : 159 - 211.

SZALLASI A 1994. The vanilloid (Capsaicin) receptor : Receptor types and species differences. *Gen. Pharmacol.* **25** : 223 - 243.

SZEBENI A, JANCSO G & WOLLEMAN M 1978. Capsaicin receptor binding. *Acta Physiol. Acad. Sci. Hung.* **51** : 13.

SZOLCSANYI J & JANCSO-GABOR A 1975. Sensory effects of capsaicin congeners I. Relationship between chemical structure and pain producing potency. *Drug Res.* **25** : 1877 - 1881.

SZOLCSANYI J 1977. A pharmacological approach to elucidation of the role of different nerve fibres and receptor endings in mediation of pain. *J. Physiol. (Paris)* **73** : 251 - 259.

SZOLCSANYI J 1982. Pungent agents producing pyrexia in Handbook of Experimental Pharmacology. Vol. 60. Pyretics and Anti-pyretics ed. by AS Milton pp 347 - 478. (*Springer-Verlag Berlin*).

SZOLCSANYI J 1993. Actions of capsaicin on sensory receptors in capsaicin in the study of Pain (Wood JN ed) pp. 1 - 26. (*Academic Press,* San Diego, CA).

TAKAKI M, JIN J-G, LU Y-F & NAKAYAMA S 1990. Effects of piperine on the motility of the isolated guinea- pig ileum : Comparison with capsaicin. *Eur. J. Pharmacol.* **186** : 71 - 77.

TANNER MJA 1979. *Curr. Top. Membr. Transp.* **11** : 279.

TENG OH, KAY JY, WEE A & LEE KO, 1998. Protective action of chill ion haemorrhagic shock induced gastric mucosal injury in the rat. **13** (10) : 1007 - 1014.

TOMINAGA M, CATERINA MJ, MALMBERG AB, ROSEN TA, GILBERT H, SKIMER K, RAUMANN BE, BASBAUM AI & JULIUS D 1998. The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron* **21** : 531 - 543.

UCHIDA M, YANO S & WATANABE K 1991. The role of capsaicin sensitive afferent nerves in protective effect of capsaicin against absolute ethanol - induced gastric lesions in rats. *Jpn. J. Pharmacol.* **55** (2) : 279 - 282.

URBAN L & DRAY A 1991. Capsazepine, a novel capsaicin antagonist, selectively antagonises the effects of capsaicin in the mouse spinal cord *in vitro*. *Neurosci. Lett.* **134** : 9 - 11.

VASILETS LA & SCHWARTZ W 1993. *Biochim. Biophys. Acta.* **1154** : 201.

VEKSLI Z, SALSBURY NJ & CHAPMAN D 1969. *Biochim. Biophys. Acta.* **183** : 434 - 446.

VINCENZI FF 1981 in The Fifth Ann. Arbour. Conference pp. 363. (*Liss, AR. Inc.* New York).

WALPOLE CSJ, WRIGGLESWORTH R, BEVAN S, CAMPBELL EA DRAY A, JAMES IF, PERKINS MN, REID DJ & WINTER J 1993. Analogues of capsaicin with agonist activity as novel analgesic: structure activity studies 1. The aromatic "A-region". *J. Med. Chem.* **36** : 2362 - 2372.

WANG JP, HSU MF, HSU TP & TENG CM 1985. *Thromb Res.* **37** : 699.

WARREN GB, HOUSLAY MO, METCALFE JC & BIRDSALL MJM 1975. Cholesterol in excluded from the phospholipid annulus surrounding an active calcium transport protein. *Nature* **255** : 684 - 687.

WATSON CPN, EVANS RJ & WATT VR 1989. Post herpetic neuralgia and topical capsaicin. Pain **33** : 333 - 340.

WEST ES, TODD WR, MASON HS & BRUGGEN JTV 1974. Blood and Other body fluids in Textbook of Biochemistry, pp. 560. (*Macmillan Pub. Co. Inc.* New York, USA).

WHITTET TD 1968. Pepperers, spicers and grocers - forerunners of the apothecaries. *Proc. Roy. Soc. Med.* **61** : 801 - 806.

WINTER J, DRAY A, WOOD JN, YEATS JC & BEVAN S 1990. Cellular mechanism of action of resiniferatoxin : A potent sensory neuron excitotoxin. *Brain Res.* **520** : 131 - 140.

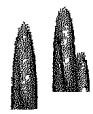
WINTER J, WALPOLE CSJ, BEVAN S & JAMES IF 1993. Characterization of resiniferatoxin binding sites on sensory neurons : Co-regulation of resiniferatoxin binding and capsaicin - sensitivity in adult rat dorsal root ganglia. *Neuroscience* **57** : 747 - 757.

WOOD AJ, VISWALINGAM A, GLUE P, ARONSON JK & GRAHAME- SMITH DG, 1989. *Clin. Sci.* **76** : 397 - 402.

YAGI T 1990. Inhibition by capsaicin of NADH- quinone oxidoreductases is correlated with the presence of energy coupling site 1 in various organisms *Arch. Biochem. Biophys.* **281** : 305 - 311.

ZAIDI A & SALEEMUDDIN M 1991. Goat erythrocyte calmodulin is not abnormal. *Indian J. Exp. Biol.* **29** : 528 - 531.

ZAIDI A AND SALEEMUDDIN M 1993. Ca^{++}-induced alterations in the activity of membrane (Ca^{++}-Mg^{++})-ATPase of human and rat erythrocytes. *Indian J. Biochim. Biophys.* **30** : 98 - 102.



8. IMPORTANT WEBSITES ON CAPSAICIN

anatomy.ucsf.edu/ohara/capsaicin.pdf

easyweb.easynet.co.uk/ gcaselton/chile/scoville.html

edgein.home.mindspring.com/science/medical.html

europa.eu.int/comm/food./fs/sc/scf/out120_en.pdf

heelspurs.com/capsaicin.html

members.tripod.com/ismhassel/recepies/rhchcaps.htm

micro.magnet.fsu.edu/phytochemicals/pages/capsaicin.html

newton.dep.anl.gov/askasci/chem99/chem99011.htm

people.cornell.edu/pages/bjm 10/capsaicin. html

people.cornell.edu/pages/bjm 10/capsaicin/capsaicin. html

pmep.cce.comell.edu/profiles/rodent/capsaicin

shop.store.yahoo.com/pharmacy/arclicfordet.html

student.biology.arizona.edu/honors98/group12pepper.html

www.aboutarachnoiditis.org/capsaicin_corticosteroid_cck_antagonists.html

www.academicpress.com/inscight/10221997/capsaic1.htm

www.aegis.com/news/bw/1998/BW980301.html

www.au.dk/en/aj2001/aj01s365.pdf

www.bagelhole.org/drafts/capsicain.html

www.banrau.de/banrau/weitere/chilli4.htm

www.capsaicin.com

www.carecure.rutgers.edu/spinewire/research/capsaicin_bhddev.html

www.cdc.gov/niosh/nmann/pdfs/5041.pdf

www.cooperfitness.com/content/support/pharmaceutical/altmed/Detail.asp

www.diabetesselfmanagement.com/article.cfm?

www.drugdebate.com/debate/thread.asp?

www.egconsult.com/scoville.htm

www.epa.gov/REDS/factsheets/4018fact.pdf

www.firegirl.com/studying/firebelly.html
www.fpnotebook.com/PHA43.htm
www.icsoffice.org
www.ijon.de/chili/scharf.html
www.jr2.ox.ac.uk/bandolier/band29/b29-6.html
www.medhelp.org
www.mohotta.com/aboutus.php
www.mohotta.com/newsletter.php
www.nlm.nih.gov/medlineplus/drug info/capsaicintopical 1202626.html
www.nursing.ivowa.edu/sites/pedspain/topicals/CAPSAIAC.htm
www.pepperworld.com/basics/capsaicin.htm
www.pepperworld.com/forrum/showmessage.asp?
www.precisionbio.com/capsaicin.htm
www.pslgroup.com/dg/21bcd2.htm
www.rsdaler.com.uk/drugs/capsaicin.htm
www.sabinsa.com/products.capscin_book.htm
www.sciential.net/capsaicin_pain_relief.htm
www.sinica.edu.tw/zool/zoolstudy
www.southernnewmexico.com/snm/capsicum.html
www.the-scientist.com/yr2000/jan/hot-000124.html
www.thinkgeek.com/stuff/apparel/3813.shtml
www.ucsf edu/pressrel/1998/03/0302caps.html
www.wellesley.edu/chemistry/Flick/molecules/capsaicin.html
www.youngagain2000.com/capsaicin.html
www/fiery-foods.com/dave/capsaicin.asp

PAPERS PUBLISHED FROM THE WORK PRESENTED IN THIS THESIS (UPTO OCTOBER 2002)

2002 : Rizvi SI and **Luqman S.** Anti-oxidative property of Capsaicin. Medicinal Chemistry Research USA. (In press)

2001 : Rizvi SI and **Luqman S.** Capsaicin-induced activation of erythrocyte membrane acetyl cholinesterase activity. Medicinal Chemistry Research USA. 10 (7/8) : 502-506.

PAPERS PRESENTED IN CONFERENCES AND SEMINARS

ORAL PRESENTATION

2000 : Nov. 3-6, 70th Annual Session of National Academy of Sciences, University of Allahabad, Allahabad.

POSTER PRESENTATION

2000 : Nov. 9-11. IInd International Symposium on Current Advances in Molecular Biology, Lucknow University, Lucknow.

2001 : Feb. 11-15, International Symposium on Current Trends in Drug Discovery Research CDRI, Lucknow.

2001 : Dec. 27-29. 70th Annual Meeting of Society of Biological Chemists, Osmania University, Hyderabad.

2002 : Jan. 3-7, 89th Indian Science Congress Association, Lucknow University , Lucknow.